TEXAS REPORTS on BIOLOGY and MEDICINE

VOLUME 14, NUMBER 4 WINTER, 1956

Copyright 1956 by the Board of Regents of The University of Texas

Contents

Page

Distribution and Effect of Radioiodinated Antithyroid Antibodies in the Injected Rat, *Ludwik Anigstein, W. B. Hardin and Dorothy Whitney* 401

Behavioral Disturbances in Animals. II. Effects on Blood Pressure, Pepsinogen, and Adrenal Weight in Rats, *Gerald A. Belkin and Alvin P. Shapiro* 415

Recent Investigations on Leukergy, *Ludwik Fleck* 424

The Effect of a Histamine Liberator (compound 48/80) on Methylcholanthrene Induced Skin Carcinoma in Mice, *P. O'B. Montgomery, T. Dillon and Andres Goth* 432

The Regularity of Egg Output of *Schistosoma haematobium*, *Carolyn M. Stimmel and J. Allen Scott* 440

Effects of the Blood Derivative, Sanguinin, on Plant Tissue, *Dorothy M. Whitney and Mario Pavan* 459

Effect of Certain Dyes on Growth *in Vitro* of Endamoeba histolytica, *Athanasius Eul Joon Yoo and G. A. Emerson* 464

Calling Attention To 558

CANCER SYMPOSIUM

M. D. ANDERSON HOSPITAL AND TUMOR INSTITUTE

Introduction, *R. Lee Clark* 473

Cancer Research Is Growing Up, *Joseph C. Aub* 475

Studies on the Enhancement of the Mutation Rate by Carcinogens, *Edgar Altenburg* 481

Metabolism in Germfree Animals, *Thomas D. Luckey* 482

Progressive Biochemical Change in the Leukemic Mouse, *Roy B. Mefferd, Jr.* 506

Carcinogenesis in the White Pekin Duck, *R. H. Rigdon* 508

Precancerous Metabolic Alterations in the Process of Azo Dye Carcinogenesis, *J. D. Spain* 528

Anticancer Activity of Plant Extracts, *Alfred Taylor, George McKenna and Henry M. Burlage* 538

Some Aspects of the Chemistry of the Pituitary Growth Hormones of Different Species, *A. E. Wilhelmi* 557

FOUNDED BY CHAUNCEY D. LEAKE, 1943

EDITOR: JOHN G. SINCLAIR

ASSISTANT EDITOR: MARY JANE STEDING

EDITORIAL BOARD:

KENNETH EARLE, ARILD HANSEN, GRACE JAMESON, ROBERT M. MOORE, WIKTOR NOWINSKI, ARTHUR RUSKIN

481
482
506
508
528
538
557

DISTRIBUTION AND EFFECT OF RADIOIODINATED ANTITHYROID ANTIBODIES IN THE INJECTED RAT*

LUDWIK ANIGSTEIN, W. B. HARDIN, AND DOROTHY M. WHITNEY

Previous studies on tracing anti-organ antibodies *in vivo* have revealed a relative accumulation of radioactivity in the homologous tissue, although extensive cross-reactions with heterologous organs were found (Anigstein *et al.*, 1954). These cross-reactions are most probably due to common antigenic components of various tissues, especially to those present in the vascular bed (Pressman and Sherman, 1951). Strong localization of kidney antibodies in adrenals and ovaries of injected rats was recently described by Bale and Spar (1954). The antigen-antibody combination upon which the localization of the specific antibody in the homologous organ largely depends, may be cytotoxic, as shown by the nephrotoxic effect of the antikidney serum (Smadel, 1936) or may induce characteristic physiological responses of the organ. In this regard, the study on the effect of antipancreas serum *in vivo* revealed hyperglycemia in the injected rat (Anigstein *et al.*, 1954). Although the mechanism of this phenomenon has not been disclosed, it is possible that the insulin present in the rat pancreas acted as one of the antigenic components of the pancreas-homogenate which elicited anti-insulin in the immunized rabbit. In this connection the recent studies of Moloney and Coval (1955) on antigenicity of insulin, and diabetes induced in guinea pig by specific anti-insulin antibodies, deserve attention.

The antigenic properties of thyroid proteins were first investigated by Hektoen and Schulhof (1923) who immunized rabbits with human, beef and swine thyroglobulin and used the antisera for precipitin tests. In studying the specificity of anti-thyroglobulin sera Hektoen and Schulhof (1927) found cross-reactions with thyroglobulins of other animal species thus indicating organ

* From the Department of Preventive Medicine and Public Health, University of Texas Medical Branch, Galveston, Texas. This work was supported by a contract (NR 130-981) between the Office of Naval Research Department of the Navy, and the University of Texas. Received for publication June 29, 1956.

specificity rather than species specificity. The degree of cross-reactions with homologous and heterologous mammalian thyroglobulins was determined by Stokinger and Heidelberger (1937) by quantitative precipitin methods. An extensive study on serological specificity of thyroid extracts was recently carried out by Witebsky, Rose and Shulman (1955). These authors used crude saline extracts of hog thyroid gland and have demonstrated by serological methods the high potency of the thyroid specific antibodies. Cross-reactions with thyroid extracts of various animals exhibited a pattern similar to that of thyroglobulins.

Materials and Methods

Preparation of the antithyroid immune sera. Thyroid tissue of white rats of both sexes (200–250 gm weight) was used for immunization of rabbits. Prior to the removal of the gland, the rats were injected intra-abdominally with Nembutal-sodium (35mg/kg) and an equal volume of heparin-sodium solution and then perfused with 1–2 liters of saline passing by gravity through a canula inserted into the abdominal aorta. The perfusate was allowed to leave by incision in the vena cava until the tissues appeared bloodless. The thyroid gland was then removed, frozen and kept in frozen state until used. The preparation of tissue suspension for injection of rabbits (3.5 lbs each) consisted in homogenizing 10–12 thyroids in 5 ml saline (average weight of one rat thyroid = 0.018 gm). For each experiment two rabbits were injected intramuscularly and intra-abdominally once a week for 4 weeks using each time 2.5 ml suspension per rabbit. Rabbits were usually bled eight days after the last injection when the serum was collected and stored at —10° C. Another immunization procedure consisted in the injection of two homogenized thyroids per rabbit by 0.1 ml intravenously and 1.0 ml intra-abdominally twice weekly for 4 weeks. Serum was collected eight days after the last injection and stored at —10° C. The serum globulin was precipitated by sodium sulphate according to the modified technique of Majoor (1945) and gamma globulin content determined by electrophoresis. The following is the detailed technique of fractionation of the euglobulins which contain the gamma portion:

To 2.0 ml of serum in a 50 ml round bottom large-neck contrifuge tube add 18 ml of solium sulfate solution (18.5/100 ml) and 25 ml ether shaking vigorously. Allow it to stand at room temperature for ten minutes. Add the ether layer detergent (Span-20) in a concentration of about 1%. Shake vigorously, centrifuge at 5,000 rpm for about 15 minutes. Upon the removal of the tubes the gamma fraction at the water-ether junction appears as a hard white wafer. Remove the "wafer" and dissolve in saline. The protein can be dialyzed against 16 liters of saline by rotary dialysis at 5–10° C for 12–24 hrs. The residue is then transferred to 20 ml saline for lyophilization. Radioiodination of the anti-thyroid gamma globulin was carried out in Abbott Laboratories, Chicago, by Gleason's technique by which 50 micrograms of potassium iodide are incorporated into 100 mg of globulin corresponding to

approx. 1 atom iodine per molecule of protein. This low iodination ratio is well within the stability limits for radio-bound protein. The specific activity of the material was approx. 32 microcuries with 1 mg globulin concentration per ml.

Rats were injected intracardially with 0.5 ml of the radioactive globulin solution in addition to 1.55 ml intra-abdominally, totaling 66 microcuries per rat.

Preparation of blood and tissue samples for radioactivity measurements. Duplicate samples of 0.2 ml of blood drawn from the heart were weighed in metal bottle caps, digested with 1.0 N NaOH and dried overnight at room temperature. For radioactivity measurements of tissues, rats were sacrificed at various intervals and perfused. Tissues were then removed, macerated and prepared in duplicate 0.2 gm samples as described for blood.

The amount of radiation emitted from radioiodine in blood and tissues was measured by the thin mica window Geiger-Mueller TGC2 tube with Tracerlab 64 scaler. Counts per minute per gram of wet tissue were corrected for background, for radioactive decay and for dosage based on 250 gm rat.

Complement fixation tests. The technique was based on procedures used and described by Witebsky et al. (1955). The rat tissues used as antigens were removed from the perfused animals and kept frozen until ready for use. Portions of the thawed organs were weighed and 10% suspensions in saline were prepared with the aid of a homogenizer. The suspensions were then centrifuged at 3,000 rpm in a refrigerated angle centrifuge for one hour. The supernate was used as antigen in both complement fixation and precipitin tests. The antisera, complement and antigen in 0.1 ml amounts were placed in refrigerator for two hours and then placed in a 37° C. waterbath for 1 hour. Sensitized cells were then added in 0.2 ml amounts and the readings made when controls became negative.

Precipitin tests. The tests were performed by adding antigen in various dilutions to the undiluted serum. After 1 hour at room temperature ring formation was observed. The tubes were then shaken and read after 24 hours in the refrigerator. Precipitin reaction was further studied by the Ouchterlony technique and agar plates were prepared according to Wilson and Pringle (1954). The antigen was placed at the apex of the triangle and the immune and normal serra placed in wells at the proper distance from the antigen. Further precipitin studies were made using the technique of Munoz and Becker (1950) designed to evaluate multiple antigen-antibody systems.

Experimental Results

Serology. The antibody titration of sera by complement fixation tests with thyroid tissue antigen revealed titers, not exceeding 1:100. There was a marked overlapping of antigenic properties in various tissues particularly in the spleen, a highly vascular organ difficult to perfuse.

The standard ring test technique for specific thyroid antibody revealed low precipitin titers. Rat antithyroid sera from rabbits

Nos. 26, 40 and 41 (pooled), 138, 139, showed titers 1:40 only after the tubes with antigen and antisera were refrigerated for 24 hours. No rings were noted at the end of one hour incubation at room temperature. The Ouchterlony technique using thyroid, adrenal, kidney, liver, testes and spleen as antigens showed a graded response. Whereas no precipitation lines were seen in the area between antithyroid serum and the thyroid antigen depot, lines were noted between this antiserum and all other tissues. The area between the antithyroid serum and the adrenal antigen showed 2 lines of precipitation, while the kidney, liver, spleen and testes antigens showed one line between antigen and antiserum. The zone between the spleen and the antithyroid serum showed an unexplained dark zone containing one precipitation line. No bands were observed between normal rabbit serum and any of the antigens tested. The precipitin test of Munoz and Becker (1950) technique gave somewhat similar results. No bands were seen with antithyroid serum and the homologous antigen, most probably due to the high dilution of the antigen, three bands appeared in the tubes containing kidney, adrenal and liver antigens, one band was seen in the tubes containing testes and spleen antigens. Tests with normal rabbit serum gave entirely negative results. A strong overlapping of the antithyroid antibodies with heterologous antigens was recorded by Witebsky *et al.* (1955). In one instance the antihog thyroid rabbit serum showed a much greater reaction with kidney extract than with the homologous thyroid extract (Witebsky *et al.*, 1955).

Radioactivity. The degree of accumulation of radioactivity *in vivo* was studied in various organs of rats injected with I 131 antithyroid gamma globulin. The respective data were expressed in terms of specific activity of tissue as related to radioactivity of blood.

The preliminary experiment was carried out in twelve rats divided in two groups of six rats each. Each rat of the test group was injected intra-abdominally with 0.75 ml. antithyroid serum, while each of the control rats was treated the same way with normal rabbit serum. Twenty-four hours later the twelve rats were given 0.5 ml. of I 131 antithyroid gamma globulin intracardially in addition to 0.75 ml. of the globulin intra-abdominally. The total of radioactivity per rat was 94 microcuries with a specific activity of 75 microcuries per mg. of gamma globulin.

A pair of rats of each group was perfused and sacrificed at the end of 24 hours and one pair of each group every other day thereafter for eight days. Radioactivity of the tissues and of the blood was measured and the tissue/blood ratios (T/B) calculated. During the first two days of observation there was a relatively higher retention of radioactivity by the thyroid of rats treated with antithyroid serum as compared with the controls injected with normal rabbit serum, but during the succeeding days the ratios reversed themselves, namely the rats pretreated with antithyroid serum began to show less radioactivity than the control with the result that on the eighth day the T/B ratio of the antithyroid series showed approximately 60% lower radioactivity than the control.

The next series of twenty-four rats, approximately 230 gm. weight, was divided into two groups of twelve each, one group injected intra-abdominally with 0.75 ml. rat antithyroid serum, (pooled from rabbits No. 40 and No. 41), whereas the control group was given the same amount of normal rabbit serum. On the following day the twenty-four rats were injected intracardially with 0.5 ml. I 131 anti-rat-thyroid rabbit gamma globulin. This material which was equivalent to 16 microcuries per rat was supplemented by intra-abdominal injection of 1.55 ml. antithyroid gamma globulin solution, totalling 66 microcuries per rat. At the end of three hours after injection, one pair of rats from each group was perfused. For eight successive days this procedure was repeated and the tissues (thyroid, adrenal, kidney, spleen, testes and liver) were prepared, by digestion and maceration, for radioactivity measurements. The first determination made during the early "dilution phase" showed approximately a three-fold concentration in the thyroid of the rats pretreated with antithyroid serum as compared with the control. A higher T/B ratio for the thyroid was still evident at the end of 48 hours during the stabilization period ("immune phase"), however beginning with the third day the radioactivity of the antithyroid pretreated rats was lower than that of the control especially on the eighth day after injection. Beginning with the tenth day the radioactivity of the blood fell to levels far below the minimum of accuracy in counting.

The analysis of these observations seems to indicate a relative suppression of radioactivity or a lower retention capacity in the

thyroids of rats pretreated with antithyroid serum as compared with the controls (Chart I, Table 1). Similarly lower radioactivity was found in the adrenals (Chart II, Table 1), the differ-

TABLE 1

Radioactivity expressed in tissue-blood ratios (T/B) for labeled anti-rat-thyroid gamma globulin injected into rats

Group A Rats pretreated with normal rabbit serum

Group B Rats pretreated with anti-rat-thyroid rabbit serum

Chart I	T/B ratios A	Day	T/B ratios B
Thyroid	66	0	220
Blood	150	2	204
	345	3	292
	411	4	368
	590	5	517
	770	6	696
	(440)	7	...
	1,000	8	835
	1,840	10	2,420
	2,230	12	15,600
Chart II	A	Day	B
Adrenal	.270	0	.039
Blood	.029	2	.487
	.298	3	.162
	.623	4	.440
	1.050	5	.502
	.810	6	.360
	.192	7	...
	.226	8	.716
	.047	10	.100
	...	12	...
Chart III	A	Day	B
Spleen	.167	0	.100
Blood	.207	2	.169
	.245	3	.126
	.145	4	.218
	.009	5	.299
	.193	6	.357
	.207	7	...
	.290	8	.348
	.290	10	.680
	.890	12	1.500

Chart IV	A	Day	B
Liver	.045	0	.460
Blood	.050	2	.053
	.057	3	.095
	.071	4	.130
	.090	5	.210
	.339	6	.328
	.063	7	...
	.182	8	.137
	.100	10	.216
	...	12	...

Chart V	A	Day	B
Testis	.082	0	.072
Blood	.135	2	.065
	.143	3	.200
	.125	4	.215
	.112	5	.168
	.123	6	.132
	.151	7	...
	(.612)	8	.103
	.173	10	.170
	...	12	...

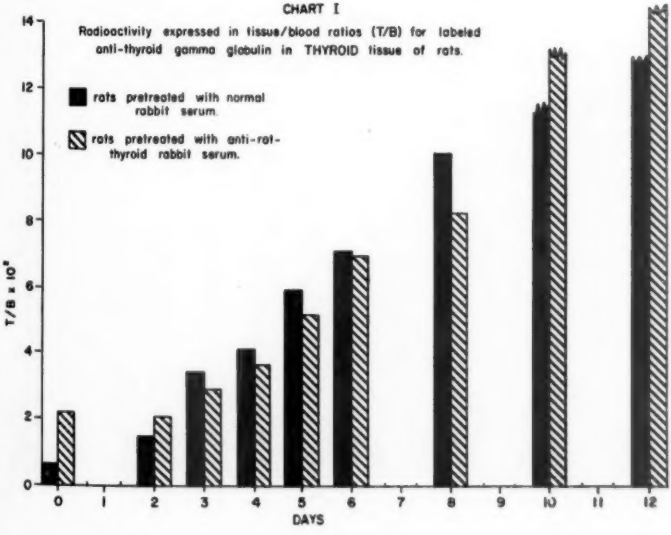


CHART II

Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-thyroid gamma globulin in ADRENALS tissue of rats.

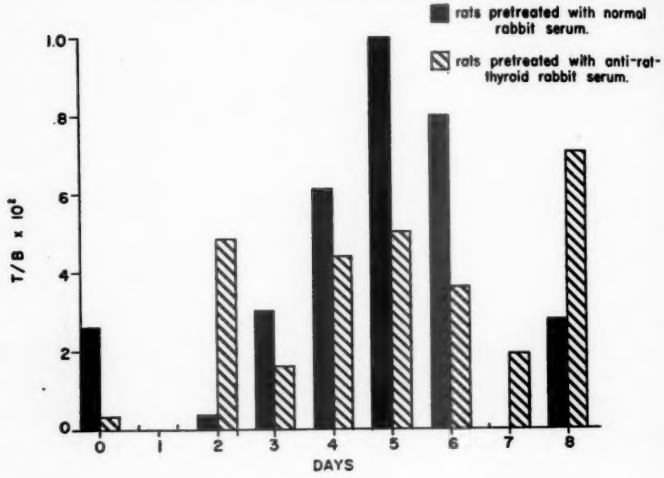


CHART III

Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-thyroid gamma globulin in SPLEEN tissue of rats.

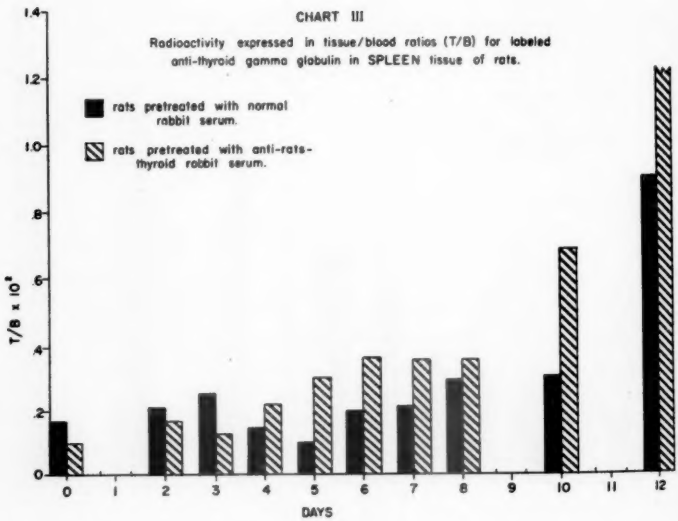


CHART IV

Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-thyroid gamma globulin in LIVER tissue of rats.

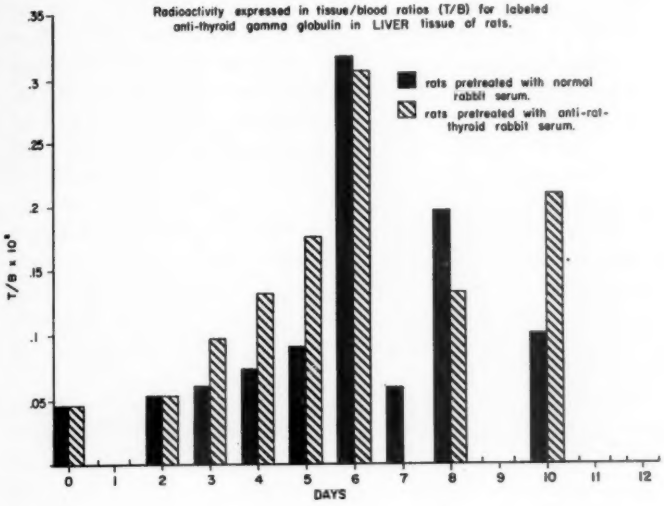
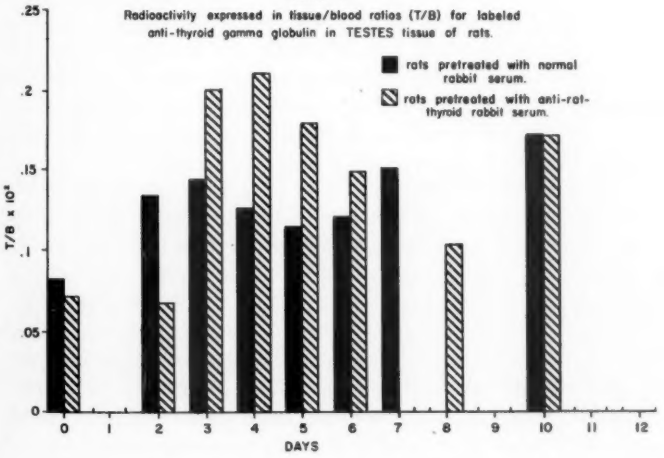


CHART V

Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-thyroid gamma globulin in TESTES tissue of rats.



ences between the T/B ratios of the two rat groups being even greater. Assuming cross-reactions of organ antibodies and the overlapping antigenicity of various organs, the fact that the adrenals of rats pretreated with the antithyroid serum show considerably lower radioactivity levels as compared with rats injected with normal rabbit serum deserves attention. On the other hand, there is a stronger localization of radioactivity in the spleen of the antithyroid series beginning with the fourth day after injection over the entire period of observation although data for the ninth and eleventh day are lacking (Chart III, Table 1). Similar results were obtained with the liver where during most of the observation period the T/B ratios were higher in the antithyroid series (Chart IV, Table 1). The T/B values for the testicular tissue were also higher at the third, fourth, fifth and sixth day of observation as compared with the radioactivity values of the control (Chart V, Table 1).

Metabolism. The physiological effect of thyroid antibodies upon the oxygen consumption of 20 day weanling rats was investigated. For this purpose twelve rats of one litter, both sexes, 20 days of age were divided in two groups (A and B) and kept in separate cages. All rats were maintained on the same diet (Purina dog chow). Group A was injected with antithyroid rabbit gamma globulin (80% pure) at a dosage of 0.2 ml. to 0.4 ml. intraabdominally, weekly or twice weekly over a period of 28 days. A total of nine injections were given to each rat of group A. The gamma globulin contained 39 mg./ml. protein with 31.2 mg. globulin per ml. of the solution. Consequently, the injected doses of 0.2–0.4 ml. correspond to 6.25–12.48 mg. globulin or a total average dose of 3.6 mg. per rat for the 28 day period.

Each group of rats was placed in a round glass desiccator jar to which a spirometer was attached (Fig. 1). Duplicate readings were taken and, following correction to standard barometric pressure and temperature, the final results were expressed in milliliters of O_2 consumed per gram/hour (Table 2). The analysis of Fig. 2 shows a steep rise in oxygen consumption curves in both groups of rats beginning with the 20th day of age. Whereas the peak of the curve in control rats is reached on the 29th day, the curve for the thyroid antiserum treated rats reaches its peak on the 32nd day and levels off lower than in the controls.

The results of these preliminary experiments seem to indicate

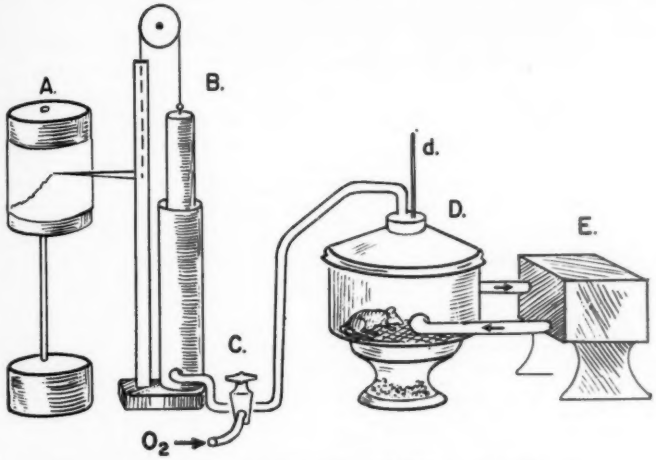


FIG. 1. *Apparatus far measurement of Basal Metabolic Rate (BMR) in rats, based on oxygen consumption.*

A. Kymograph recording drum
B. Spirometer with oxygen
C. Three way stopcock which fills the cylinder with O_2 and allows the oxygen to flow to:
D. Desiccating jar where the rat is placed; d. thermometer
E. Electric motor for air circulation. Moisture is absorbed by CaO at the bottom of D.

that the antithyroid gamma globulin repeatedly injected into weanling rats exerts a delaying action on the rate of oxygen consumption as compared with the control series.

Histological examination of the rat thyroid was based on specimens from weanling rats previously used for basal metabolism measurements. Sections were prepared from thyroids of weanling rats after a course of injections of antithyroid gamma globulin and compared with the thyroids of untreated rats. In the thyroids of the "globulin series" the follicular cells were found hypertrophied reaching far into the colloid indicating an increase in the thyroid hormone. Another thyroid of the same series shows a

TABLE 2

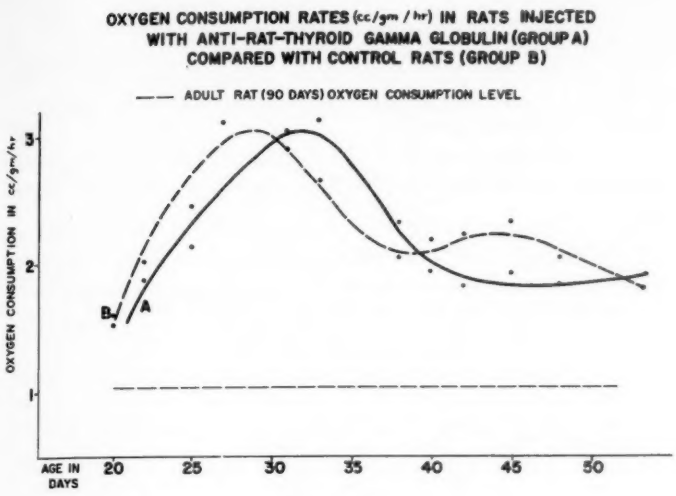
Oxygen consumption rates (O_2cc gm/hr) in rats injected with anti-rat-thyroid gamma globulin (group A) and in control rats (group B)

Age days	O_2		Weight	
	A	B	A	B
20	1.53	1.59	25.5	24.0
21	...	...	29.0	25.0
22	2.02	1.85	30.5	29.6
25	2.10	2.43	38.8	35.0
26	...	...	43.0	38.3
27	1.92	3.06	47.0	43.0
29	...	...	52.4	48.6
31	3.08	2.88	67.0	61.3
33	3.10	2.61	76.0	69.6
34	...	...	82.0	75.6
35	...	...	87.5	80.6
38	2.30	2.01	101	93.6
39	...	...	105	96.6
40	1.91	2.16	109	100
41	...	...	108	...
42	1.90	2.17	118	108
45	1.90	2.30	129	120
46	...	...	135	125
47	...	...	137	128
48	1.71	2.04	144	130
52	1.93	1.80	162	157

Age given in days since birth.

Weight given in grams averaged from the combined weight of each group of six rats.

reactive hyperplasia undergoing degeneration and resembling carcinomatous changes. Also in this gland extreme activity is evident changing the histological structure completely. The thyroid of the control rat shows "pseudo-giant' cell formation due to degeneration of the epithelial cells of the acinis. On the whole, no remarkable differences were noted between the thyroids of rats injected with anti-rat-thyroid antibodies as compared with those of the controls. Extreme hyperplasia (hypertrophy) dominates the picture being at the same time associated with degenerative changes. No definite conclusions can be drawn from these limited observations, but the subject deserves further study.



Summary

The rat thyroid was used for testing the action of specific antibodies and for evaluation of the responses of the organ to these antibodies. Antithyroid immune sera were prepared in using rat thyroid tissue as antigen for immunization of rabbits. The antibody titration of immune sera with thyroid tissue antigen revealed antibodies of low titer with cross-reactions against other rat organs. For tracing the distribution of thyroid antibodies in the injected rat, radioiodinated gamma globulin of the antisera was used. The thyroid of rats pretreated with antithyroid serum and subsequently injected with radioactive gamma globulin showed less radioactivity than the thyroid of rats pretreated with normal rabbit serum. Weanling rats 20 days of age, were repeatedly injected with antithyroid gamma globulin and their oxygen consumption curves compared with those of control rats. Whereas the peak of the oxygen consumption curves in normal control rats was reached at their 29th day since birth, the oxygen curve in the series of rats pretreated with thyroid antibodies reached its peak after a 72 hours delay. These observations together with the lower capacity of the thyroid to retain the injected thyroid antibodies after pretreatment with specific antiserum, seem to indicate a functional suppression of the thyroid gland.

Acknowledgement

Thanks are due to Mr. Bruce D. Genereaux of the Tissue Metabolism Laboratory for his valuable assistance in the preparation of the gamma globulin.

The authors are indebted to Dr. J. Finerty of the Department of Anatomy for making the BMR apparatus available for these studies.

BIBLIOGRAPHY

Anigstein, L., et al., Distribution of radioiodinated anti-organ antibodies in the injected rat. *Tex. Rep. Biol. & Med.*, 12: 945–959, 1954.

Bale, W. F. and I. L. Spar, In vivo localization of rat organ antibodies in ovaries, adrenals and other tissues, *J. Imm.*, 73: 125–133, 1954.

Hektoen, L. and K. Schulhof, The precipitin reaction in thyroglobulin. *J. Am. Med. Assoc.*, 80: 386, 1923.

Hektoen, L., H. Fox, and K. Schulhof, Specificness in the precipitin reaction of thyroglobulin, *J. Inf. Dis.*, 40: 641–646, 1927.

Majoor, C. L. H. The possibility of detecting individual proteins in blood serum. *Yale Jour. Biol & Med.*, 18: 419–441, 1945–46.

Moloney, P. J. and M. Coval. Antigenicity of insulin: Diabetes induced by specific antibodies, *Bioch. Jour.*, 59: 179–185, 1955.

Munoz, J. and E. J. Becker. Antigen-antibody reaction in agar, *J. Immun.*, 65: 47–58, 1950.

Pressman, D. and B. Sherman. Immunological specificities and cross-reaction in vascular beds of liver, kidney and lung. *Fed. Proc.*, 10: 416, 1951.

Rose, R. and E. Witebsky. Studies on organ specificity. II. Serological interrelationship among thyroid extracts of various species, *Jour. Immun.*, 75: 282–290, 1955.

Smadel, J. Experimental nephritis in rats induced by injection of anti-kidney serum, *J. Exp. Med.*, 64: 921–942, 1936.

Stokinger, H. E. and M. Heidelberger. VI. The reaction between mammalian thyroglobulins and antibodies to homologous and heterologous preparations, *J. Exp. Med.*, 66: 251–272, 1937.

Wilson, M. W., and B. H. Pringle. Experimental studies of the agar-plate precipitin test of Ouchterlony, *J. Immun.*, 73: 232, 1954.

Witebsky, E. et al. Studies on organ specificity. I. The serological specificity of thyroid extracts, *J. Immun.*, 75: 269–281, 1955.

BEHAVORIAL DISTURBANCES IN ANIMALS. II. EFFECTS ON BLOOD PRESSURE, PEPSINOGEN, AND ADRENAL WEIGHT IN RATS*

GERALD A. BELKIN, AND ALVIN P. SHAPIRO†

Since emotional disturbances produce acute physiological changes in man and animals, it has been postulated that chronic diseases such as peptic ulcer, hypertension, and hyperthyroidism may result when these disturbances are prolonged. The contrasting sympathetic and parasympathetic responses demonstrated by Cannon (1923) in these acute phenomena have been extrapolated to explain the involvement of specific organs following chronic anxiety (Alexander 1950), and the proposal of the General Adaptation Syndrome following stress has suggested a possible pathogenesis (Selye 1947).

However, there have been few attempts to test these hypotheses in an experimental animal model. Although disturbances of behavior in animals have been investigated extensively, more studies are needed in which these disturbed states are correlated with physiologic malfunction and chronic pathological changes, in order to clarify psychosomatic theory.

The etiologic relationships between chronic anxiety and hypertensive vascular disease are particularly difficult to investigate without an experimental model. Acute changes in blood pressure in man occur readily following emotional disturbances and clinically affect the course of the disease and its response to therapy (Shapiro, 1956), but these influences have never been demonstrated decisively to produce chronic vascular changes. Farris (1945) and Rothlin (1953) have produced high blood pressure in rats by audiogenic stimulation, but the techniques require considerable physical stimulation and significant vascular disease has not developed in these animals.

We have undertaken studies to devise an experimental model

* From the Department of Internal Medicine, The University of Texas, Southwestern Medical School, Dallas. Supported by United States Public Health Service Grants M-739 and H-1954. Received for publication June 15, 1956.

† Present address: Department of Clinical Science, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

in which the effects of emotional disturbances on blood pressure and related functions may be defined precisely. By varying the types of psychological disturbance and by employing measures intended to potentiate damage to the vascular system in these experiments, we have attempted to evaluate the relative importance of psychologic and anatomic abnormalities in the etiology of hypertensive vascular disease. Studies in cats exposed to the Masserman technique for establishment of experimental neuroses have been reported previously (Shapiro 1955). The present report describes results in rats in whom the effects of a simple form of behavioral disturbance and the potentiating influence of a high sodium intake have been investigated.

Methods and Materials

Fifty male Sprague-Dawley rats, weighing approximately 200 grams each, were divided randomly into four groups. Cages were cleaned and rotated every two weeks. All animals were fed rat chow* ad lib supplemented by fresh lettuce and carrots. The four groups of animals were treated as follows:

Cage	No. of animals	Stress	Drinking fluid
A	13	no	distilled water
B	13	no	1.5% saline†
C	12	yes	distilled water
D	12	yes	1.5% saline†

* Uncle Johnny Mills, Houston, Texas.

† With 1.5% saline as drinking water, rats drink approximately twice as much fluid as controls receiving distilled water. This supplies 1 to 1.5 gms of salt per day, and constitutes approximately a 10% salt diet. For reasons pointed out in the discussion, this is close to the maximum amount of salt a rat can be given when it is supplied in drinking water.

The rats were weighed and systolic blood pressure measured weekly. The latter was determined with the tail plethysmograph, with the animal anesthetized lightly with ether (Williams 1939). Weights and blood pressures were not significantly different in the four groups prior to the experiment.

In order to produce behavioral disturbance (hereafter referred to for descriptive purposes as Stress), each animal was placed in a runway, two feet in length and divided into three compartments by eccentrically placed doors. The top of the runway was plate glass; the floor consisted of a grid, constructed of ¼" aluminum rods, placed ¼" apart, and traversing the length of the runway. An automatic timing device and a variable voltage regulator permitted electrification of one end and the center compartment, or the other end and the center, alternately, at 25 second intervals. A buzzer (conditioned stimulus) wired to the top of the runway sounded for three seconds every 25

seconds, and was followed, beginning during the third second, by electrification of the grid with a charge of approximately 30 volts. This was sufficient only to jolt the animal mildly. To escape from the charged end to the safe end of the runway, the rat was compelled to run through the charged middle compartment; 25 seconds later, he was forced to shuttle back. He could escape the electrical shock by responding to the conditioned stimulus.

Sufficient runways, all wired to the same circuit, were available to stimulate 12 rats simultaneously. The animals were placed in the runways for a thirty minute period, during which time they made approximately thirty round trips of the apparatus. Immediately thereafter, the rats were locked into one of the compartments for a six hour period during which the buzzer alone sounded at 25 second intervals. They were allowed access to food and the appropriate fluids during this period.

The apparatus thus accomplished the following: 1) the rat was exposed to a noxious stimulus (non-convulsive electric shock) from which it could escape by responding to a conditioned stimulus; 2) it was forced to repeat this maneuver at 25 second intervals, returning for safety each time to the previously harmful compartment; 3) after a short period of this behavior (30 minutes), the animal was exposed for a longer period (6 hours) to the conditioned stimulus, with escape barred but without the noxious stimulus. These maneuvers were carried out three times weekly for 10 weeks*, at which time, following a two day rest period, all animals were sacrificed. They were anesthetized with ether and exsanguinated by cardiac puncture. Stomach and duodenum were opened and examined grossly for evidence of ulceration. Heart, kidneys, and adrenals were removed, cleaned of extraneous tissue, and weighed immediately in tared containers on a Sartorius balance accurate to 0.1 mg. Tissues were fixed in 10% formalin and sections stained with hematoxylin-eosin. Oil red O stains of frozen sections of adrenal also were prepared (Gormori 1952). Plasma pepsinogen was determined by the method of Mirsky et al. (1952).

Results

I. *Behavior*

In pilot studies, the rats learned the avoidance response as long as it was unidirectional only. When shuttle activity was introduced however, this response would break down, so that reinforcement with the noxious stimulus again became necessary. During the course of the experiment, about one third of the animals in both groups would develop the avoidance response during a given Stress period; this was usually extinguished by the next period. Different rats displayed the response on each Stress day; only a few were consistent responders. However, all

* Physical stress was thus quite minimal; actually the animals were forced to run and be exposed to the non-convulsive shock for only 1.5 hours per week.

animals continued to display a "startle" reaction to the conditioned stimulus. This took the form of a slight jump and/or the striking of a "catatonic" positioning and posturing. When so arranged, the animals did not move unless vigorously prodded.

The over-all behavior of the animals was unaffected. They remained docile, except for one or two animals in each group who intermittently displayed biting activity at the conclusion of a thirty-minute period of stimulation. In respect to behavior, significant differences between animals on 1.5% saline solution or water were absent.

II. *Physiological changes*

These data were subjected to analysis of variance and are summarized in Tables 1 and 2.

A. Weight gain

Weight gain was least in the group receiving saline and Stress. Stress alone, but not saline alone, interfered significantly with weight gain. Gross differences in food intake among the groups were not noted; however, balance studies were not performed.

B. Blood pressure

Although a slightly higher systolic blood pressure was noted in the group receiving saline alone as contrasted with the group receiving saline and Stress, analysis of variance revealed that all groups were from the same population as regards this variable.

C. Adrenals

A highly significant increase in adrenal weight in proportion to body weight was demonstrated in the groups receiving Stress. Saline alone produced no changes and did not potentiate the changes of Stress.

D. Heart and kidneys

Heart and kidney weights were increased significantly in proportion to body weight, in the groups receiving 1.5% saline. Stress exerted no effect and was not potentiated by sodium.

E. Plasma pepsinogen

Plasma pepsinogen values were not significantly different between the groups.

TABLE 1

Treatment	Weight gain (gms)	Systolic blood pressure (mm. Hg)	Heart weight (mg/100 gms)	Kidney weight (mg/100 gms)	Adrenal weight (mg/100 gms)	Pepsinogen (mcg tyrosine/cc. plasma)
Control (C)	148±23	115±10	278±18	679±64	13.5±1.5	251±67
1.5% saline (Na)	134±27	119±7	300±21	749±62	13.2±1.2	298±83
Stress (St)	126±16	115±10	288±17	684±48	15.9±2.3	321±98
1.5% saline + Stress (Na + St)	108±18	110±6	301±27	738±49	16.5±1.6	309±100
F value (between groups)	7.54	2.80	3.47	4.79	11.47	1.52
"p"	<.01	NS	.05>.01	.01	<.01	NS

TABLE 2

Treatment	Weight t*	Weight P	Adrenals t*	Adrenals P	Heart t*	Heart P	Kidneys t*	Kidneys P
C × Na	1.67	NS	0.5	NS	2.7	<.01	3.1	<.01
C × St	2.6	.05>.01	3.4	<.01	1.2	NS	0.21	NS
C × Na + St	4.6	<.01	4.8	<.01	2.7	<.01	2.5	.05>.01
Na × Na + St	3.0	<.01	5.2	<.01	0.13	NS	0.47	NS
St × Na + St	2.0	NS	0.8	NS	1.4	NS	2.2	.05>.01

$$*t = \frac{\bar{x}_1 - \bar{x}_2}{V\sqrt{\frac{1}{\tilde{n}_1} + \frac{1}{\tilde{n}_2}}}; \text{ where } V = \sqrt{\text{within sample variance}}$$

III. *Pathological changes*

With the Oil red O stains of the adrenal, lipids were diminished in the zona glomerulosa of the "salted" animals, with and without Stress, despite the fact that the adrenal weights did not differ significantly from those in the "unsalted" group. The heart, kidneys, and adrenals of the "salted" animals demonstrated no lesions, grossly or microscopically, nor were any changes produced by Stress. No ulcers were seen in the stomach or duodenum of any animals, although it has been stated that these may occur spontaneously in rats (Hay, 1942).

Discussion

The technique employed to produce behavioral disturbance provided a stimulus of pain and fear, from which the rat was permitted to escape, but in so doing submitted to additional discomfort. Subsequently, the animal was exposed repeatedly to the conditioned stimulus for these disturbances, but escape was blocked. Although it is not intended to analogize these anthropomorphic conceptions of rat psychology to man in any precise way, the experimental design in the rat provided a situation reminiscent of certain conflicts in man in which feeling states such as hostility and frustration are engendered.

The psychostimulation evoked a reaction of stress in the rats as measured by adrenal hypertrophy but was not associated with

additional physiological or anatomical changes other than slight retardation in growth. Moreover, a high sodium intake did not exacerbate the stress reaction, although it was itself responsible for an increase in weight of the heart and kidneys and a decrease of lipids in the zona glomerulosa of the adrenal (Bacchus 1950). Disturbances in gastric function or pathologic changes in the stomach were not elicited.

The failure to produce hypertensive vascular disease may lie in the type, intensity, and duration of psychological disturbance employed, in the lack of a properly prepared target organ, or indeed in the fact that behavioral disturbances are unimportant in the etiology of the ailment. The lack of gastro-intestinal disturbance similarly has a number of implications regarding organ specificity in psychosomatic diseases. However, it would seem unwise to speculate further concerning these problems from experiments that were intended primarily as pilot investigations of methodology. Rather, the studies should be considered only as providing empirical data to guide further investigations.

One of the major problems in research in chronic psychosomatic disorders is the lack of such psychophysiological data from which hypotheses may be formulated and the absence of experimental models in which these hypotheses can be tested. Present theories are not soundly based and consequently may be misleading. For instance, our data indicate that the theory of the General Adaptation Syndrome (GAS) may not be pertinent to hypertensive disease. An adrenal cortical stress reaction apparently was invoked, and yet no hypertension, vascular disease, or any other "disease of adaptation" developed, even with potentiation by a high sodium diet. Similarly, empirical data may clarify and allow more precise formulation of the concepts of organ specificity in psychosomatic disease.

The absence of vascular disease in animals receiving 1.5% saline as drinking fluid deserves additional comment. Development of hypertension in rats receiving 2% saline as drinking water has been previously reported (Sapirstein 1950) and in pilot studies in our laboratory this was also noted, but the rats rapidly succumbed. With free access to distilled water, the rat can tolerate large amounts of sodium in his diet, as has been demonstrated by Richter (1954). However, when the excess sodium is given in the drinking fluid in concentrations significantly over 1.5%, it

can be calculated from Richter's data that the animal is unable to make available sufficient fluid for body needs over and above that needed to excrete the excess salt. The 1.5% saline drinking fluid therefore, apparently serves merely to "hypertrophy" mechanisms responsible for sodium excretion and may increase sodium and water content of the tissues, but yet does not produce the vascular changes which Meneely (1953) has described in rats on diets high in sodium-containing foods.

Summary

An experimental model for the investigation of psychosomatic disorders, particularly hypertensive vascular disease, was studied. In the rat, ten weeks of a specific type of psychostimulation produced adrenal hypertrophy, but blood pressure did not increase. Administration of 1.5% saline as drinking fluid did not produce vascular changes nor potentiate the stress reaction. The experiment demonstrated that use of an animal model may supply empirical data which will permit more specific formulation of current hypotheses concerning psychosomatic disorders.

REFERENCES

Alexander, F. *Psychosomatic Medicine*. New York: W. W. Norton and Co., 1950.

Bacchus, H. Cytochemical study of the adrenal cortex of the rat under salt stress. *Am. J. Phys.*, 163: 326, 1950.

Cannon, W. B. *Bodily Changes in Pain, Hunger, Fear, and Rage*. New York: Appleton & Co., 1923.

Farris, E. J., E. H. Yeakel, and H. S. Medoff. Development of hypertension in emotional gray Norway rats after blasting. *Am. J. Phys.*, 144: 331, 1945.

Gomori, G. *Microscopic Histochemistry*. University of Chicago Press, Chicago, 1952.

Hay, L. J., R. L. Varco, C. F. Code, and O. H. Wangenstein. Experimental production of gastric and duodenal ulcers in laboratory animals by intramuscular injection of histamine in beeswax. *Surg., Gyn., and Obst.*, 75: 170, 1942.

Meneely, G. R., R. G. Tucker, W. J. Darby, and S. H. Auerbach. Chronic sodium chloride toxicity: hypertension, renal, and vascular lesions. *Ann. Int. Med.*, 39: 991, 1953.

Mirsky, I. A., P. Futterman, S. Kaplan, R. H. Broh-Kahn. Blood plasma pepsinogen. I. The source, properties, and assay of proteolytic action of plasma at acid reactions. *J. Lab. Clin. Med.*, 40: 17, 1952.

Richter, C. P., and H. D. Mosier. Maximum sodium chloride intake and thirst in domesticated and wild Norway rats. *Am. J. Phys.*, 176: 213, 1954.

Sapirstein, L. A., M. L. Brandt, and D. R. Drury. Production of hypertension in the rat by substitution of hypertonic NaCl solution for drinking water, *Proc. Soc. Exp. Biol. & Med.*, 73: 82, 1950.

Selye, H. A. The general adaptation syndrome and disease of adaptation. *J. Clin. Endocrin.*, 6: 117, 1947.

Shapiro, A. P., and P. Horn. Blood pressure, pepsinogen, and behavior in rats subjected to experimental production of anxiety, *J. Nerv. Ment. Dis.*, 122: 222, 1955.

Shapiro, A. P. Consideration of multiple variables in the evaluation of hypotensive agents, *J.A.M.A.*, 160: 30, 1956.

Williams, J. R., T. R. Harrison, and A. Grollman. A simple method for determining blood pressure in the rat, *J. Clin. Invest.*, 8: 393, 1939.

RECENT INVESTIGATIONS ON LEUKERGY†

LUDWIK FLECK*

The characteristic features of leukergy consist in the appearance in the circulating blood of certain leukocytes which exhibit adhesiveness and tendency to agglomerate (Fleck and Murczynska, 1947; 1949: Fleck, 1951). Leukergy‡ occurs in all instances of accelerated leukopoiesis, *i.e.* in various inflammatory states, in febrile infectious diseases, in normal pregnancy, after profuse bleeding as well as after intensive muscular activity. Leukergy can be provoked artificially in rabbits by intravenous injection of bacteria, pyrogenic substances or Menkin's "leukocytosis promoting factor." Intrapleural injection of turpentine will also produce leukergy. In man, leukergic response has been noted after lumbar air insufflation and is supposed to be of central origin. The leukergic leukocytes originate from the bone marrow, and they undergo a series of changes during the circulation, and perform special functions.

Our more recent investigations have proceeded chiefly along two lines: 1) demonstration of the immunological role of leukergy, 2) establishment of the relation between the properties of leukergic leukocytes and their metabolism.

The concept of leukergy which denoted no more than the mere clumping of the white cells in the above mentioned states, took on a broader meaning, namely activation of the leukocytic system, characterized by the production of adhesive leukocytes rich in enzymes and in energy-rich material displaying an intensive immunological activity. The leukergic agglomeration of leukocytes is not a serological phenomenon and has no connection with

* Head of the Department of Microbiology, Mother and Child Institute, Warsaw, Poland, Kasprzaka, 17.

† Edited by Ludwik Anigstein and Dorothy Whitney. Received for publication July 1, 1956.

‡ The test for detecting leukergy and determining its degree is as follows: A citrated blood sample (one part 3.8% sodium citrate solution to four parts blood) is incubated for three hours. A large drop is placed on a slide, rocked gently and after drying in the incubator, is stained with methylene blue without fixation. From this the percentage of agglomerated leukocytes is determined.

the autoagglutination of leukocytes as described by many authors. The leukergic leukocytes cannot be simply termed "inflammatory leukocytes" since they may appear in conditions with no relation to inflammation, *e.g.*, after blood loss.

The immunological role of leukergy results from the following properties of leukergic leukocytes: 1) greater motility than normal leukocytes, 2) phagocytic activity exceeds several times that of the normal leukocytes, 3) adhesiveness, causing the shifting of leukocytes to the infection foci as well as to the blood depots (lung, spleen, liver). The shifting to the lungs is of particular significance in antibacterial defense since bacteria which have invaded the circulation are trapped and intensively phagocytized even in the small pulmonary vessels and may be subsequently eliminated together with the phagocytes into the alveolar spaces.

For estimation of motility of leukocytes the slide-cell technique of Martin *et al.* (1950) was used. The distance between the centrifuged erythrocytes and the border line of leukocytic migration after 20 hours served as migration index. The indices of normal animals were compared with those of animals injected with turpentine, with killed *B. coli* or with Menkin's "leukocytosis promoting factor." The results are shown in Table 1.

TABLE 1

Number of experiments	Leukergy in % of clumped leukocytes	Mean number of leukocytes in cubic mm.	Mean migration index	Mean migration index/ 1,000 cells
17	0 to 5	8,105 ± 38*	1.6 ± 0.12	200μ
6	5 to 40	11,833 ± 100	2.6 ± 0.34	220μ
19	40 to 80	13,178 ± 831	3.3 ± 0.28	250μ

* Standard error of the mean = $\sqrt{\frac{\Sigma (d^2)}{n (n-1)}}$

The difference in the mean migration indices for negative leukergic blood (0 to 5%) and weakly positive (5 to 40%) is 1 ± 0.36, thus indicating a noticeable difference. Still more statistically significant are the differences between negative and strongly positive leukergic blood (40% to 80%), 1.7 ± 0.29. The migration index per 1,000 cells is 250μ, for positive leukergy as compared with 200μ for aleukergic cells.

A double or triple dilution of leukergic blood with its own plasma will not reduce the mean migration index below that of

aleukergic blood although the mean number of leukocytes per cubic millimeter falls to one half of the mean number of leukocytes in aleukergic blood (Table 2). The value of the migration index undoubtedly depends on the number of leukocytes, however, the greater motility of leukergic leukocytes which has been clearly demonstrated must be emphasized.

TABLE 2

Leukergy 0% to 5%		Leukergy 40% to 80%					
		Undiluted blood		Blood diluted 1:2		Blood diluted 1:3	
Mean migration index	Mean leukocytosis	Mean migration index	Mean leukocytosis	Mean migration index	Mean leukocytosis	Mean migration index	Mean leukocytosis
1.6	8,105	3.3	13,178	2.03	6,589	1.7	4,292

We have studied the phagocytic power of rabbit leukocytes for *Staphylococcus albus* before the injection of killed *B. coli* or Menkin's "leukocytosis promoting factor," in comparing the phagocytosis of aleukergic and leukergic animals. We used as opsonin the same serum, namely the serum taken before the injection of the rabbit to avoid the interference due to the opsonin level changes. The results are shown in Table 3.

TABLE 3

Number of experiments	Mean phagocytic index before leukergy	Mean phagocytic index during leukergy	Difference of means	Remarks
8	1.45 ± 0.22	9.38 ± 1.7	7.93 ± 1.7	37°C. incub. temp.
6	11.47 ± 1.9	31.65 ± 5.04	20.18 ± 5.38	39°C. incub. temp.
5	34.32 ± 9.8	106.7 ± 17.6	72.3 ± 20.2	39°C. incub. temp.
8	4.07 ± 0.7	6.81 ± 1.5	2.77 ± 1.59	surface phagocytosis temp. 39°C.

The percentage of granulocytes which did not phagocytize was 6.4 in aleukergic blood as compared with 1.6 in leukergic blood, thus four times lower.

The effect of leukocytosis in leukergy is enhanced by clumping of blood platelets which arrest bacteria (Rickenberg's phenomenon) and deliver them to the leukergic leukocytes. These leukocytes unlike aleukergic leukocytes have the property of adhering to the platelets.

Direct determination of bactericidal activity of leukergic and aleukergic blood to which an equal number of staphylococci were added, showed that aleukergic blood gave several times more colonies than did leukergic blood. The results are shown in Table 4.

TABLE 4

Number of rabbits	Leukergy negative		Leukergy positive			
			Blood undiluted		Blood diluted 1:2	
	MLN*	MCN†	MLN	MCN	MLN	MCN
9	9,788	238.5	13,722	88.1	6,935	134

* MLN = mean leukocyte number. † MCN = mean colony number.

From leukergic blood samples diluted with their own plasmas 1:2, *i.e.*, from those samples where the leukocyte number was diminished by one-half, but without reduction of the anti-bacterial serological potency, we obtained a mean number of colonies smaller than from aleukergic blood, but greater than from undiluted leukergic blood. This proves that the greater bactericidal power of leukergic blood (experimentally determined) is caused neither by an increase of serological forces during leukergy, nor by merely the increased leukocyte-number in leukergic blood, since this number is smaller in diluted leukergic blood than in the aleukergic blood.

By inducing artificial leukergy we successfully protected rabbits, in three series of experiments, against lethal doses of virulent staphylococci. In a similar way, we also prolonged the life of animals infected with pneumococci.

Another mechanism important for antibacterial defense and connected with leukergy is the accumulation in certain places of leukocytes due to their adhesiveness. Many authors have described the effect of leukocytic adhesiveness on margination in the vessels, and on diapedesis from the vessels (A. Krogh, W. Barry Wood, Ch. A. Stetson, and others).

According to earlier (Levaditi, 1901) and recent publications (A. S. Weisberger, 1951; V. Menkin, 1952; H. R. Bierman, 1952), the cause of leukopenia immediately following an intravenous injection of bacteria or other substances such as glycogen, india ink, etc., is the accumulation of leukocytes in the pulmonary circulation. Bacteria and other foreign particles introduced

into the veins are also trapped there. We succeeded in demonstrating that stickiness plays an important role in arresting the leukergic leukocytes in the lungs. First and foremost such leukocytes disappear from the greater circulation and accumulate in the pulmonary circulation. They can transitorily be brought back into the general circulation by an adrenalin injection. The retention of leukocytes in the lungs is of immunological importance. In this region, an intensive phagocytosis takes place and subsequent elimination of bacteria together with the leukocytes via pulmonary alveoli and bronchi. We demonstrated this effect by injecting tubercle bacilli intravenously, proving after one hour in impression smears of lung tissue their accumulation and phagocytosis in this organ. After 24 hours in inflammatory foci, *e.g.*, in the peritoneal cavity after an irritant injection or in pus, almost all leukocytes are leukergic. The same holds true for inflammatory accumulations of leukocytes in the liver or spleen. The proof can be furnished by cytochemical methods.

Ehrlich, followed by Best (1903), established the presence of increased amounts of glycogen in pus cells, in the leukocytes of exudates and those of the blood in infection leukocytosis. In recent years many authors (R. Wagner, 1947; M. Wachstein, 1949; W. S. Beck, 1953; W. N. Valentine, 1953; R. Robineaux, 1954) have written on this subject. Increased amounts of phosphatase in leukocytes from an infectious disease and from exudates are mentioned by these authors.

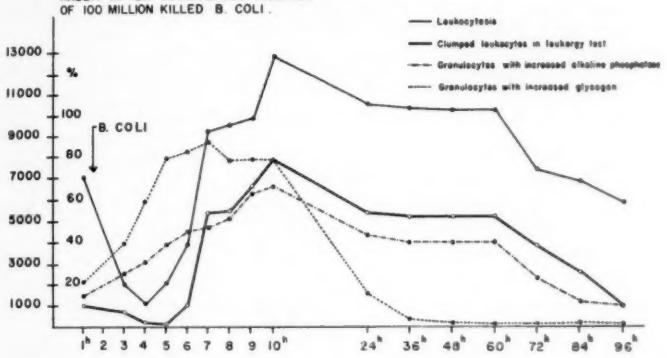
These data prompted us to investigate the relation between leukergy and glycogen as well as the alkaline phosphatase content in the leukocytes. We used cytochemical methods after Hotchkiss (glycogen) and Gomori (alkaline phosphatase). The results of the experiments on rabbits are shown in table 5.

The leukopenic stage appears soon after the injection of bacteria (20 to 40 minutes), subsequently (2 to 4 hours later) the leukocytosis stage begins, which lasts about 3 to 4 days. The leukergy test becomes positive simultaneously with the beginning of leukocytosis or a little sooner. About 2 hours before the positive leukergy test, the percentage of glycogen containing cells increases, this state lasting only 10 to 18 hours. The increase of the percentage of phosphatase positive cells begins later than that of glycogen positive cells, the interval being about 30 minutes to one hour. It lasts until the positive leukergy test vanishes.

The glycogen and alkaline phosphatase reactions of the bone marrow, after bacterial injection, precede the reaction in the blood one half to one hour, beginning 20 to 30 minutes after the stimulus. In rabbits, we find the described glycogen and phosphatase reactions taking place in all leukergy cases: after bacteria, pyrogenic substances, "leukocytosis promoting factor" injections, after blood loss, following intrapleural administration of turpentine etc. In pus and exudates, we find all cells to be strongly positive.

TABLE 5

LEUKOCYTIC RESPONSE CURVES OF A RABBIT AFTER INTRAVENOUS INJECTION OF 100 MILLION KILLED B. COLI.



In man, the difference between leukergic and aleukergic leukocytes, as far as glycogen in concerned, is less than in rabbits, but still distinct.

The reaction as a whole and the order of its successive stages suggests that very soon, after the stimulus, the leukopoietic organs deliver into the circulation peculiar energetic leukocytes rich in glycogen and enzymes (glycogenolysis) which produce within two to three hours the factor responsible for the positive leukergy test. Their glycogen content diminishes within a few hours, but the stickiness remains. The changes of the blood picture both at the beginning and at the end of leukocytosis, are determined by the appearance and disappearance of these peculiar leukocytes.

It is unlikely that the leukergic state represents a stage in the

life cycle of ordinary granulocytes because there is no concurrence in the disappearance of the stickiness and granulocytosis. If this were true, the positive leukergy test would disappear and the granulocytes would remain in the circulation, but this never happens. In any case, the leukergic leukocytes preserve *in vivo* and *in vitro* their adhesiveness for the duration of life.

It seems permissible to look at leukergy as a special defense mechanism, promptly working in infection as well as in some cases of stress (pregnancy, wounds with blood loss, shock).

REFERENCES

Beck, Wm. S., and Wm. N. Valentine: The Carbohydrate Metabolism of Leukocytes: A Review, *Cancer Research*, 13: 309–317, 1953.

Bierman, Howard R., et al.: Studies on Cross Circulation in Man. I. Methods and Clinical Changes, *Blood*, 6: 487–503, 1951.

Bierman, Howard R., et al.: The effect of Intravenous Histamine Administration on the Level of the White Blood Count in the Peripheral Blood, *Blood*, 6: 926–935, 1951.

Comroe, J. H., Jr.: *The Functions of the Lung.* Harvey Lectures, p. 110, 1952–53.

Dausset, J.: Presence D'Une Leuco-Agglutinine dans Trois Serums de Malades Leucopeniques, *Le Sang*, 24: 410–417, 1953.

Fleck, L.: Le Phenomene de la Leukergie et la Possibilitie de L'Utiliserdans L'hematologie, *Le Sang*, 20: 1–4, 1949.

Fleck, L.: Über Leukergie, *Acta Haematologica*, 7–8: 282–293, 1952.

Fleck, L.: Leukergia. Postepy, *hig. med. do dosn*, 4: 7–43, 1951.

Fleck, L., Szczygielska, J.: The Immunological Significance of Leukergy. I. *Annales Univ. M. Curie-Sklodowska*, 5: 507–521, 1950.

Fleck, L., and J. Szczygielska: The Immunological Significance of Leukergy. II. *Annales Univ. M. Curie-Sklodowska*, 5: 523–533, 1950.

Fleck, L., and Z. Murczynska: Leukergy: Clumping of White Blood Cells in Cytologically Homogenous Groups. A New Phenomenon of Inflammation. *Tex. Repts. on Biology and Medicine*, 5: 156–167, 1947.

Fleck, L.: Further Investigations on the Phenomenon of Leukergy, *Tex. Repts. on Biology and Medicine*, 9: 697–708, 1951.

Fleck, L., and D. Borecka: The Leukergy Reaction in Various Diseases. *Annales Univ. M. Curie-Sklodowska*, 1: sec. D: 335–349, 1946.

Fleck, L., and I. Lille-Szyszkowicz: Leukergy and Glycogen Content in Leukocytes. *Bull. de L'Academie Polonaise des Sciences*, 3: 137–143, 1955.

Goudsmit, R., and J. J. Van Logham: Studies on the Occurrence of Leucocyte-Antibodies, *Vox Sanguinis*, 3: 58–67, 1953.

Krogh, H.: *The Anatomy and Physiology of Capillaries.* Yale Univ. Press, 2nd rev. ed., 1929.

Lissac, J., G. Mathe, M. Borron, and J. Bernard: Contribution Experimentale A L'etude du Role Du Poumon Dans La Regulation Leucocytaire, *Le Sang*, 26: 501–509, 1955.

Martin, S. P., C. H. Pierce, Gardner Middlebrook, and Rene J. Dubos: The Effect of Tubercle Bacilli on the Polymorphonuclear Leucocytes of Normal Animals, *Jour. of Exp. Med.*, 91: 381–393, 1950.

Menkin, V.: *Newer Concepts of Inflammation.* Thomas. Springfield, 1950.

Menkin, V.: L'homeostase dans l'inflammation. *Schweiz. Med. Wochen.* 82: 1184–1186, 1952.

Robineaux, R.: Movements Cellulaires et Function Phagocytaire des Granulocytes Neutrophiles, *Revue d'Hematologie*, 9: 364–402, 1954.

Stetson, C. A., Jr.: Studies on the Mechanism of the Schwartzman Phenomenon. Certain Factors Involved in the Production of the Local Hemorrhagic Necrosis, *Jour. Exp. Med.*, 93: 489–505, 1951.

Valentine, W. N., J. Folette, and J. S. Lawrence: The Glycogen Content of Human Leukocytes in Health and in Various Disease States, *Jour. Clin. Inves.*, 32: 251–258, 1953.

Wachstein, M.: The Distribution of Histochemically Demonstrable Glycogen in Human Blood and Bone Marrow Cells, *Blood*, 4: 54–60, 1949.

Wagner, R.: Studies on the Physiology of the White Blood Cell, *Blood*, 2: 235–243, 1947.

Weisberger, A. S., R. A. Guyton, R. W. Heinle, and J. P. Storaakli: The Role of the Lungs in the Removal of Transfused Lymphocytes, *Blood*, 6: 916–935, 1951.

Wood, W. B., M. R. Smith, W. D. Perry, and J. W. Berry: Studies on the Cellular Immunology of Acute Bacteremia, *Jour. Exp. Med.*, 94: 521–534, 1951.

THE EFFECT OF A HISTAMINE LIBERATOR (Compound 48/80) ON METHYLCHOLANTHRENE INDUCED SKIN CARCINOMA IN MICE

P. O'B. Montgomery, T. Dillon, and Andres Goth*

In 1923 Borrel, Boez and de Coulon (1) drew attention to the pronounced dermal mast cell reaction obtained by painting mouse skin with tar. This reaction was particularly intense beneath a benign papilloma induced by the tar. This pronounced mast cell reaction to dermal carcinogen application has since been repeatedly confirmed (3, 5, 6, 7). More recently Cramer and Simpson (2) have reconfirmed the work of the past investigations and made the additional observation that the mast cell hyperplasia was a response which occurred only in the areas of epidermal hyperplasia but was not associated with epidermal neoplasia. In view of this relationship between the application of dermal carcinogen and mast cell reaction, it seemed of interest to study the effect of compounds which are known to disrupt mast cells on carcinogenesis induced by methylcholanthrene.

It has been demonstrated in recent years (8) that mast cells store and release histamine. In addition, it has been shown by the same investigators that a variety of compounds, referred to as histamine liberators can localize in the cytoplasm of mast cells and can cause a disruption of these cells. In 1954 Fawcett (4) reported that intraperitoneal injections of compound 48/80‡ depleted rat mast cells of their granules and concomitantly produced a rise in the histamine content of the peritoneal fluid. This observation coupled with the previous reports associating dermal mast cell hyperplasia with the response to dermal applications of carcinogens led us to perform a preliminary experiment on

* From the Departments of Pathology and Pharmacology, The University of Texas Southwestern Medical School, Dallas, Texas. This project was supported by a grant from the U. S. Public Health Service through the National Advisory Arthritis and Metabolic Disease Council.

‡ A mixture of dimers, trimers, and tetramers resulting from a condensation of N-methychomanesylamine and formaldehyde. This compound was kindly supplied to us by Dr. E. J. de Beers, the Wellcome Research Laboratories, Tuckahoe, New York.

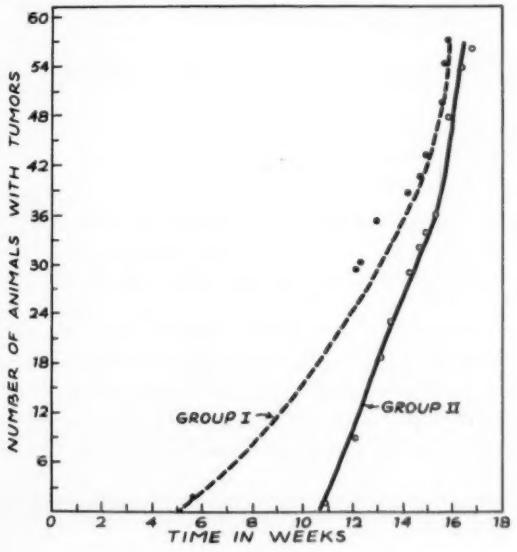
the effect of long continued injections of 48/80 on mice simultaneously subjected to dermal applications of methylcholanthrene in acetone. This preliminary experiment indicated that the carcinogenic effect of methylcholanthrene could be accelerated by the simultaneous administration of 48/80. More definitive experiments were then carried out and it is the purpose of this paper to describe the results of these latter experiments.

Materials and Methods

One hundred and sixty young adult female Swiss mice were used in the experiment. The back of each mouse was shaved and to this area 3 drops of .5% methylcholanthrene in acetone were applied each Monday, Wednesday and Friday. The mice were divided into two equal groups, and group I received daily intraperitoneal injections of 50 micrograms of compound 48/80 in saline, while Group II served as a control. (Preliminary experimentation with 30 mice had shown that 75 micrograms of 48/80 given intraperitoneally killed the majority of the animals). The above regimen was carried out in both groups, and all animals examined daily by two examiners until a grossly malignant lesion of the skin appeared. At this time the animal was sacrificed

TABLE 1

The time of occurrence of carcinoma in Group I and Group II



and the day recorded. The skin of the back was removed and fixed in 95% ethyl alcohol. Following this blocks were cut through gross areas of neoplastic tissue and paraffin sections made. These sections were stained for 4 hours with Toluidine blue at pH of 2. They were air dried and mounted with permount. The gross diagnosis of squamous cell carcinoma of the skin was confirmed in each case, and no errors in gross diagnosis were found. In addition the sections were studied for their mast cell content and distribution while the individual mast cells were compared for their granule content and general cytologic characteristics.

Results

Table 1 is a graph depicting the number of animals with tumors plotted against the time of occurrence of the tumors in weeks. Prior to the eleventh week only three animals showed malignancies. Two of these animals were in group I and one animal was in group II. All of the animals in group I developed malignancies by the end of the sixteenth week and by the nineteenth week all of the animals in group II had developed malignancies.*

An X^2 analysis of a four-fold table was made of the results, and these X^2 values with their corresponding P values are shown in Table 2. An example of this analytical method is shown below:

	With cancer	Without cancer	
Group I	12 (a)	54 (d)	
Group II	1 (c)	72 (d)	
	13	126	139 = N

$$X^2 = \frac{N\left(cbc\text{-}ad\right) - \frac{N}{2}^2}{(a+b)\ (c+d)\ (a+c)\ (b+d)}$$

The mast cells in the skin were studied in all of these mice in the original Groups I and II and these results were compared with the mast cells of mice from the following additional control groups.

Group 1: 15 normal young adult female Swiss mice; Group 2: 48 young adult female Swiss mice given one intra-peritoneal injection of 50 micrograms of 48/80 and sacrificed in lost of 12 at the end of 1 hour, 4 hours, 8 hours, and 24 hours; Group 3: 48 young adult female Swiss mice were divided into two lots and

* 12 animals in Group I, and 19 in Group II died of unknown causes during the course of the experiment and were cannibalised so that no tissues were taken.

treated with methylcholanthrene and 48/80 in accordance with the original experiment. The animals in Group 3 were sacrificed in equal numbers at the end of 1 week, 2 weeks, 3 weeks, and 4 weeks. The study of these control mouse skins in relation to the experimental skins revealed the following facts: No differences in the mast cell structure, the number of mast cells or their distribution could be attributed to the drug 48/80.

TABLE 2

DAY	PERCENTAGE *		X^2	P
	Group I	Group II		
77	14.3	1.2	9.68	<1%
88	33.4	11.1	13.64	<1%
91	35.7	12.3	14.39	<1%
100	45.3	29.7	3.65	<5%
107	51.7	43.3	3.47	<5%
112	65.5	57.0	5.43	<2%

$$*\text{PERCENTAGE} = \frac{\text{No. of animals with tumors}}{\text{No. of animals starting exp.}}$$

The number of animals developing carcinoma in Group I and Group II with their time of occurrence and corresponding P values

Although no differences in the mast cells or their number and distribution could be attributed to the effects of 48/80, there were differences between the normal mouse skin content of mast cells and their distribution when compared to the mast cells in the skins painted with methylcholanthrene. In these latter skins there were significant increases in the number of mast cells in the areas of skin hyperplasia induced by methylcholanthrene. Figure 1 shows the mast cell distribution in the normal mouse skin. Here there are one to three mast cells randomly distributed through the dermis in every low power field. Figure 2 shows a section of epidermal hyperplasia in a mouse painted for 15 weeks with methylcholanthrene. Here the increase in the number of dermal mast cells is striking. Figure 3 shows the mast cell distribution in relationship to a mass of neoplastic squamous cells. Here one sees a marked dearth of mast cells adjacent to or intermingled with

the nest of neoplastic squamous cells. These observations were quite consistent in all of the mouse skins.

Discussion

The observation that dermal mast cells increased when the skin of a mouse was painted with tar is generally credited to Borrel *et al.* Borrel was the first protagonist of the viral theory of cancer and the mast cell aggregates which he noted were interpreted by him to indicate that the tar application had made the skin susceptible to infection by neoplastic agents probably of viral origin. In a more definitive study Cramer and Simpson demonstrated the observations made here (2). Namely that mast cells increased in number when methylcholanthrene was applied to the skin of mice, and that there was a direct relationship between such mast cell hyperplasia and the epidermal hyperplasia induced, but that there was an inverse relationship to the develop-

FIG. 1. Mast cell of normal mouse skin.

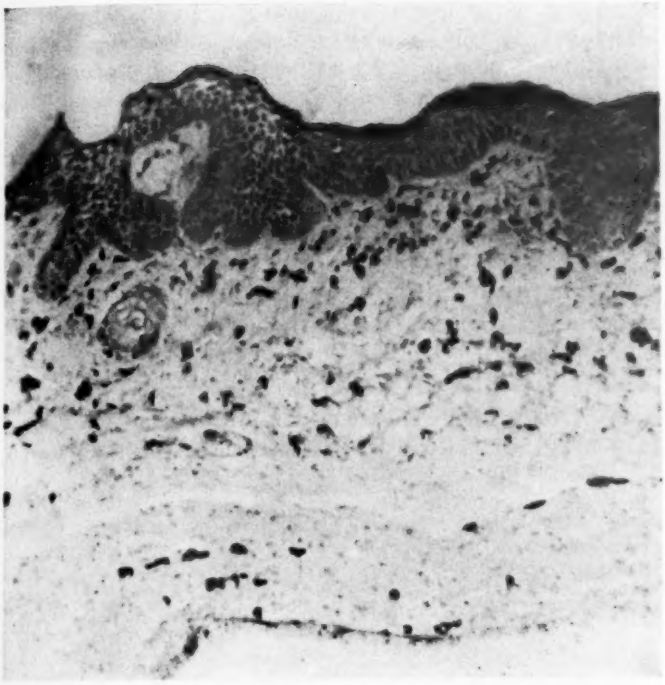


FIG. 2. Mast cell hyperplasia associated with epidermal hyperplasia.

ment of squamous cell carcinomas. In fact, mast cells were almost totally absent in or about neoplastic masses of cells. In a more recent paper Sylven (9) has shown that acetone application to the mouse skin is without effect on mast cells. They likewise noted a hyperplasia of mast cells three weeks after methylcholanthrene application. This effect appeared to be independent of the acetone solvent or of the absolute per cent of the methylcholanthrene in the solvent.

It is interesting to note that we were able to detect no differences in the mast cell number, distribution or morphology within the various groups studied, which we felt we could relate to the action of 48/80. Thus 48/80 injected mice and normal mice appeared the same with our technic for mast cell study. Methylcholanthrene painted mice and similar mice simultaneously in-

jected with 48/80 appeared the same in regard to their dermal mast cells. These observations are in contrast to those of Fawcett (4). He observed that 48/80 in tyrode's solution produced lysis of mast cells in the rat mesentery when the mixture was given intraperitoneally. This lysis was accompanied by a simultaneous rise in the histamine content of the tyrode solution. It may well be that the rat mast cell and the mouse mast cell differ greatly in their response to 48/80. It may also be that our technics and their time of application were sufficiently crude so that no histologic data could be obtained.

Further studies are now in progress to determine the role of histamine and heparin in methylcholanthrene induced skin tumors of mice.

Summary

1. The effect of prolonged injection of 48/80 into female Swiss mice being simultaneously painted with methylcholanthrene has been observed.

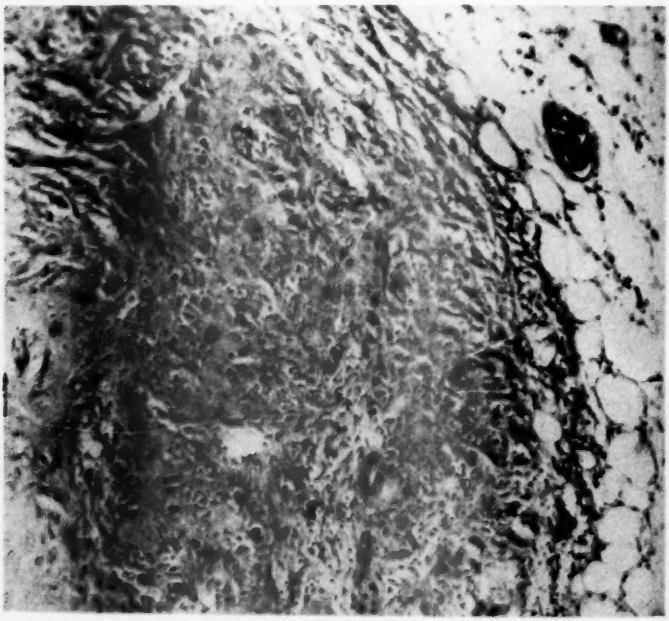


FIG. 3. Squamous cell carcinoma showing relative absence of mast cells.

2. Hyperplasia of dermal mast cells was a feature of the epidermal hyperplasia induced by methylcholanthrene.

3. When neoplasia supervened on the hyperplastic skin dermal mast cells were conspicuous by their absence from the neoplastic masses.

4. No histologically demonstrable effects on mast cells; either in relation to single cell structure, or numbers and distribution of cell aggregates, in any of the groups studied could be attributed to the injections of 48/80.

5. Under the conditions of these observations methylcholanthrene induced skin tumors appeared at a significantly accelerated rate in receiving daily intraperitoneal injections of compound 48/80.

6. These experiments suggest certain relationships between mast cells and carcinogenesis and should provide the basis for an exploration of the possible role of mast cell products, such as histamine and heparin, in the development of experimental skin tumors.

REFERENCES

1. Borrel, A., L. Boex, and A. de Coulon: Cancer du gourdron chez la souris, *Comp. rend. Soc. de biol.*, 88: 402–406, 1923.
2. Cramer, W., and W. L. Simpson: Mast cells in experimental skin carcinogenesis, *Cancer Research*, 4: 601–616, 1944.
3. Dreifuss, W., and B. Block: Uber die diinstliche Erzengung von metastasierenden Mause-carcinomen durch Bestandteile des Teerpeches. Liinische und histologische Untersuchungen, *Arch. f. Dermat. u Syph.*, 140: 6–63, 1922.
4. Fawcett, D. W.: Cytological and pharmacological observations on the release of histamine by mast cells, *J. Exp. Med.*, 100: 217–224, 1954.
5. Lipschiitz, B.: Untersuchungen iiber die Entstchung des experimentellen Teercarcinoms der Maus. *Ztschr. f. Krebsforsch*, 21: 50–97, 1924.
6. Peyron, A.: Sui certains elements lympho-con'onctifs du tissu sous-cutane de la Souris et leur presence dans l' epithelioma experimental du goudron., *Comp. rend. Soc. de biol.*, 88: 151–154, 1923.
7. Regand, C., and A. Lacassagne: A propos des mastocytes des epitheliomas. Importance de la fixation pour la coloration des granulations des mastocytes, *Comp. rend. Soc. de biol.*, 87: 1084–1086, 1922.
8. Riley, J. F., and G. B. West: The presence of histamine in tissue mast cells, *J. Physiol.*, 120: 528–537, 1953.
9. Sylven, Bengt, and L. G. Larsson: The mast cell reaction in mouse sk'n to some organic chem'cals. III. The early effect of aromatic hydrocarbons, *Cancer Research*, 8: 449–464, 1948.

THE REGULARITY OF EGG OUTPUT OF *SCHISTOSOMA HAEMATOBIUM**

CAROLYN M. STIMMEL AND J. ALLEN SCOTT

An apparent revival of interest in the quantitative aspects of schistosome infection has stimulated belated analysis of data on the egg output of *Schistosoma haematobium* collected in Egypt in 1931. Relatively little has been published on the subject in the intervening years. Scott (1938) showed that the egg output of *S. mansoni* was no more irregular than that of hookworm in the same persons nor than that of hookworm and other intestinal worms reported by other authors. Barlow (1949) stated that in infections of *S. haematobium* there is a "uniform number of eggs appearing in daily urinations," a statement which was apparently based on preliminary analysis of the data to be reported here. Gerritsen *et al.* (1953) studied the output of *S. haematobium* eggs in a series of 12 hour consecutive specimens and concluded that the variability was large.

As far as we can determine, the present study deals with the longest series of observations on record in which the collection of successive specimens was under strict control. The variation from specimen to specimen was high and associated with rather regular fluctuation within the daily cycle. The variation between the total eggs produced on successive days was much less. Emphasis has been placed on analysis of variability of egg counts in relation to their use for estimating the severity of infections. Some attention has been given to the problem of diagnosing the presence or absence of infection. Some evidence is also provided with regard to the anatomical and physiological factors involved in the elimination of eggs.

Opinion is varied as to the mechanism by which eggs get into the lumen of the bladder from the venule in the wall where the

* Contribution from the Department of Preventive Medicine and Public Health and the Laboratory of Helminth Research, The University of Texas Medical Branch, Galveston. This paper was condensed from a thesis presented by Miss Stimmel in partial fulfillment of the requirements for the degree of Master of Arts. The data were collected by Drs. Claude H. Barlow and J. Allen Scott under the auspices of the Rockefeller Foundation. Received for publication August 3, 1956.

female worms are usually located. Brumpt (1930) and van den Berghe (1937) dicuss this matter but offer little information specifically applying to *S. haematobium*. Their emphasis on the effect of contraction of the bladder would seem to be supported by our data. Kohlschütter and Koppisch (1941) showed that in *S. mansoni* infections the eggs adhere to the endothelium of the venule. Endothelial cells migrate or proliferate over them, excluding them from the lumen of the venule. Some of these eggs remain in the tissues surrounded by an inflammatory reaction, but others migrate through the tissues to the mucosa and eventually reach the intestinal lumen. Presumably the process may be similar in infections of *S. haematobium* in the bladder. Barlow (1949), however, hypothesized that the female worm may penetrate the wall of the venule and lay eggs in cysts. As the miracidia develop they may exude a histolytic substance dissolving the tissues, and the cyst is presumably ruptured by the contraction of the bladder at urination. The data presented here will be shown to provide evidence in support of some hypothesis involving rupture of cysts or extrusion from the mucosa by mechanical action of contraction at urination. Otherwise these data do not provide evidence as to the exact mechanism and the details will therefore not be discussed further.

Materials and Methods

The subjects were two laboratory workers with a secondary school education who understood the purpose of the experiment and co-operated willingly. There is good evidence, which is supported by analysis of the data, that virtually all specimens passed during the period were presented. Both cases were at the time of the experiment living under conditions in which they were no longer exposed to infection. Case 1, a 15 year old male, was infected with *S. haematobium* but showed no symptoms of the infection. The study was begun on March 12, 1931. The total urine output for each 24 hour period was collected in a single container during the first 13 days of the study. For the next 31 days the subject was instructed to divide collections into night and day periods, the night period including the time following a urination at 1600 hours through the first morning specimen, usually passed at about 0630 hours. The specimens passed following the first morning specimen through one at 1600 hours constituted the day period. For the final 10 days of the study he was instructed to urinate according to normal habits, passing each specimen into a separate container and recording the time of passage. Case 2, a 16 year old male, was infected with both *S. haematobium* and *S. mansoni* but the eggs of the latter species were always very few in number. The records analyzed here include only *S. haematobium* eggs passed in the

urine. He showed minimal symptoms of the infection. The study of this case began on May 15, 1931 and continued for 10 days, following the same schedule as did Case 1 for the final 10 days.

The specimens were processed in the laboratory with a modification of the procedure described by Barlow (1931). The contents of each container were stirred with a stirring instrument known to provide thorough and even dispersal of the eggs, then two 15 ml. portions were transferred to centrifuge tubes and centrifuged at low speed. The urine supernate was then removed from each tube without disturbing the sediment which was spread in an enclosed area on one or more microscope slides. All eggs from each 15 ml. portion were carefully counted by two experienced technicians. The urine was measured and the total number of eggs in each specimen estimated by appropriate calculations.

The variability of the counting method has been subjected to statistical analysis. When the counts of the eggs made by the two technicians on the same tube did not agree closely, the counts were rechecked by the laboratory supervisor. The variability from this source was therefore negligible. The variability between the accepted counts on the two tubes of urine from each specimen was analyzed by the method used by Scott (1938). The coefficient of variation of the average or sum of the counts of two parallel tubes based on the difference between them, without considering variation between specimens, was 16 per cent for the 44 specimens passed by Case 1 during the last 10 days. Since this variability is much less than the variability between specimens or between any grouping of specimens, it seems reasonable to conclude that the method was adequate.

The amount of urine passed by the subject is related to the climatic conditions. The 10 day periods in which separate specimens were collected are the most critical parts of the study in this respect. At the time of year when these observations were made the mean temperature in Cairo is about 24° C. and the mean relative humidity about 50 per cent. The diurnal variation of temperature is about 15° C. and of humidity about 30 per cent.

Presentation of Data

The detailed data of this study are given in a thesis by Stimmel (1956) which is available in the University of Texas Library. The data most essential for interpretation of the findings are presented below.

Information from the data on the two cases studied has some bearing on several aspects of schistosome infection. Clinicians are frequently interested in the reliability of examination of the dejecta in determining the presence or absence of infection. These data add a little to the information now available on this question. Epidemiologists are interested in the same question but are also concerned with the possibility of using egg counts to

estimate the average intensity of infection in a group of people without resorting to an exhaustive clinical examination. Since helminths generally do not multiply in the definitive host, one can assume that there will be some correlation between the number of worms present and the damage they do. This correlation will not often represent a straight line relationship and will vary with the chronicity of the infection. There is also a correlation between the number of worms present and the number of eggs they produce, but this correlation has been shown in the intestinal nematodes to depend on the age of the worms and various other factors. In the case of schistosomiasis there is the further factor that not all of the eggs reach the exterior, a factor again affected by the degree of chronicity. On these various problems the present data provide little or no information. Before they can be studied properly, however, still another problem must be faced, namely that the count of eggs at one examination may not reliably represent the level of egg output over a period of time. On this latter problem the present data have a direct bearing.

The most stable estimate of the number of eggs produced is the average daily output taken over a long period of time, providing that within that period no general change of level occurs. In this study we have available such an average based on 54 consecutive days in one case and on 10 days in the other. In practice it is seldom possible to obtain a count of more than one day's output. Our data provide an estimate of the reliability of any one day's total in comparison with the long time average. More often the most that can be obtained is a specimen representing one complete passage of urine. Sometimes only a portion of such a specimen can be obtained. The question also arises as to whether some particular specimen, such as the first morning passage, is more reliable than a random specimen. Information on these points is available from an analysis of the daily cycle of egg output in these two cases.

The Average Daily Egg Output

The average egg output of Case I over a period of 54 consecutive days was 8,440 eggs per day, and for a 10 day period of Case II was 50,497 eggs per day. The 54 daily totals for Case I are shown in figure 1. The nearly horizontal line represents the

trend of the egg output with increasing time as calculated from the daily totals. The equation for this line is $Y = 8{,}052 + 14X$, where X is the time in days and Y the daily egg output. The slope of this line is not significantly different from zero as shown by a "t" test, therefore it is clear that the level of egg output did not change significantly during this period. Similar data for the 10 day study of Case II will appear in later tables. No trend was

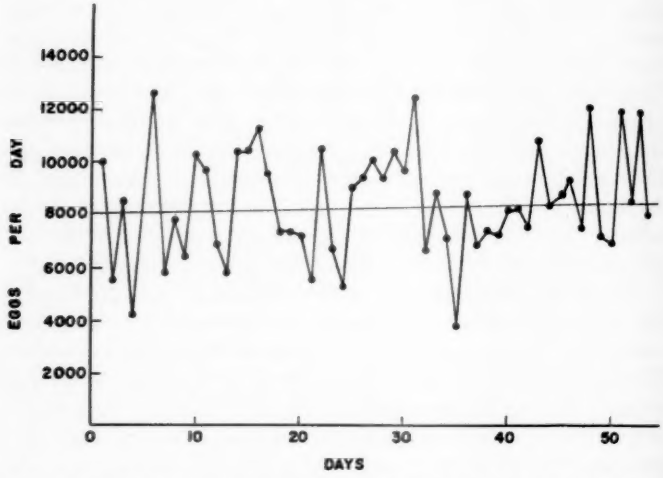


FIG. 1. Total daily egg output for the 54 day period of Case 1 and straight line fitted to the trend of the daily averages.

detectable in the shorter period in this case. It is obvious from figure 1 that there was considerable variation in the daily totals of Case I, the lowest being about 4,000 eggs and the highest about 12,500. The coefficient of variation for the series was 24 per cent. Similar variation appears in the daily totals for Case II, which range from 32,500 to 69,000 eggs, with a coefficient of variation of 28 per cent. At first sight this variation impresses one as being quite high. It actually is much lower than the variability of egg output of most of the cases of intestinal nematodes which have been studied (Scott, 1938) and egg counts with these species have proven to be of great practical value in epidemiological studies.

The relatively long series of observations on Case I provides an opportunity to study some of the factors related to this variability in daily egg output. The principal question concerns correlation between the egg output and the volume of urine. The mean daily urine volume was 1,221 ml. with a coefficient of variation of 22 per cent. The correlation coefficient between daily egg output and daily urine volume is +.378, which is statistically significant when treated with Fisher's "z" transformation. The estimating equation is $Y = 4{,}919 + 2.88\ X$, where Y is the egg output and X the urine volume. Even though the correlation coefficient is significant, the resulting coefficient of determination indicates that only 14 per cent of the variability in egg output is associated with variation in urine volume leaving 86 per cent of the variation to be explained by other factors. Some of these factors will be discussed later.

Since Case II was studied for only 10 days the use of correlation analysis has less validity. The mean daily urine output was 753 ml. with a coefficient of variation of 21 per cent. The correlation coefficient between daily egg output and daily urine volume was —.784 and was significant at the 5 per cent level. The regression equation was $Y = 101{,}638 - 68X$ but it is doubtful if much meaning should be attached to those figures in such a short series. It seems safe to say at least that they support the conclusion that most of the variability in daily egg output is not explained by the variation in urine output.

The mean eggs per milliliter of urine, calculated from the total daily egg output and total urine volume, is 7.11 eggs per ml. with a coefficient of variation of 27 per cent for Case I, and 73 eggs per ml. with a coefficient of variation of 44 per cent for Case II. Since these coefficients of variation are higher than those for total egg output, the use of eggs per unit volume of urine on a daily basis would have no advantage over the use of total eggs per day.

Egg Output for Night and Day Periods

It was suggested at the time of the study that one factor in this variability in egg output might be related to the subject's relative inactivity at night. Consequently, Dr. Barlow suggested that for Case I, beginning with the 14th day, a comparison be made of

night and day periods divided as previously mentioned at 1600 and 0630 hours.

The mean urine output for the 31 night periods was 649 ± 21 ml. as compared to the mean of 566 ± 32 ml. for the 31 shorter day periods. The mean egg output, on the other hand, was only 2,058 ± 157 eggs for the night period as compared with 6,383 ± 308 eggs for the day period. Expressing these results in terms of eggs per unit volume of urine resulted in a mean for the night period of 3.26 ± 0.27 eggs per ml., and 11.79 ± 0.55 eggs per ml. for the day period. There was a significant positive correlation between total eggs and urine volume for the day periods but no similar correlation for the night periods. Through the use of analysis of covariance the night and day means for egg output were adjusted for regression and multiplied by the appropriate factor to produce estimates of 24 hour totals. These estimates were 3,407 eggs per day when based on the night period, and 16,061 eggs per day when based on the day period, compared to the actual 24 hour mean count of 8,840 eggs. It is obvious that neither of these values would be a good estimate.

It is clear, then, that real differences existed between the egg output at night and that during the day. The most obvious explanation seems to be that the differences are somehow related to differences in the degree of activity of the individual.

The Diurnal Cycle of Egg Output

At the time the data were being collected preliminary analysis of the data from night and day collections indicated this definite relationship to activity which has just been mentioned. There was also an indication that additional information would be needed for elucidating the factors involved. Consequently separate specimens passed according to the subjects' natural habits were collected for 10 days for Cases I and II. The resulting data are presented in table 1.

When the number of eggs per specimen and the urine volume per specimen for the two cases were plotted on a time scale, a diurnal cycle was noted. Three typical days are shown in figure 2 for Case I and in figure 3 for Case II. The most obvious conclusions are that the urine volume is typically highest in the first morning specimen, as might be expected, and that the eggs

TABLE 1

Urine volume and egg output per specimen for Cases I and II over a 10 day period. The days were adjusted to begin after the first morning specimen for Case I and before the first morning specimen for Case II.

		CASE I				CASE II	
Day	Hour	Urine volume in ml.	Total eggs in specimen	Day	Hour	Urine volume in ml.	Total eggs in specimen
45	0910	265	477	1	0630	90	6,860
	1130	345	1,507		1140	155	8,644
	1405	235	3,071		1330	55	23,313
	1715	140	2,221		1830	200	13,770
	0640	465	1,782		1930	100	5,333
46	0955	115	2,204	2	0540	260	2,696
	1218	335	3,026		0930	125	1,608
	1425	160	1,147		1330	165	10,846
	1912	295	2,753		1530	100	17,489
	0615	460	521		1650	195	9,142
47	0954	305	1,149		1920	110	6,630
	1025	175	1,563	3	0540	250	6,157
	1430	275	2,475		1110	125	11,556
	1830	130	2,422		1345	160	16,853
	2345	355	533		0005	175	5,386
	0550	500	900	4	0700	260	2,572
48	0935	275	2,447		1050	90	9,425
	1414	245	5,267		1325	55	20,021
	2130	335	2,948		1400	15	11,989
	0520	670	1,206		1540	50	12,113
49	1200	265	5,388		1830	100	7,822
	2010	275	1,265		2100	100	5,142
	0540	330	825	5	0630	160	2,244
50	1110	230	5,114		1110	120	4,339
	2015	210	1,204		1330	50	18,177
	0425	600	320		1550	60	8,240
51	0725	390	702		1620	40	14,047
	1145	270	4,995		2040	130	1,189
	1315	70	2,133		2300	170	1,500
	2125	340	1,768	6	0600	325	995
	0555	355	1,112		1135	140	3,445
52	0915	145	3,147		1620	220	16,115
	1345	120	3,700		2030	140	8,567
	0600	485	1,681		2330	125	7,703
53	1100	300	5,120	7	0630	230	1,290
	1800	735	1,568		1020	190	1,191
	2015	495	4,488		1305	115	20,516
	0425	505	135		1800	225	11,312

TABLE 1—Continued

Urine volume and egg output per specimen for Cases I and II over a 10 day period. The days were adjusted to begin after the first morning specimen for Case I and before the first morning specimen for Case II.

		CASE I				CASE II	
Day	Hour	Urine volume in ml.	Total eggs in specimen	Day	Hour	Urine volume in ml.	Total eggs in specimen
54	0925	370	3,207		2350	180	2,441
	1435	205	2,481	8	0700	175	5,481
	2355	465	2,155		1155	120	11,832
	0555	615	717		1800	120	30,582
					2130	100	18,945
				9	0630	160	2,210
					1140	130	6,185
					1610	125	39,057
					1900	110	8,183
					2140	85	11,293
				10	0600	185	1,192
					1055	175	1,597
					1440	100	13,208
					2015	275	10,173
					2355	115	6,351

per specimen are low at this same time. After the first morning specimen, the succeeding urine specimens during the day tend to decrease in size, generally reaching a minimum early in the afternoon. The volume curve then tends to rise for the remainder of the 24 hours, reaching a maximum after the sleeping hours. The egg output in terms of eggs per specimen shows an almost exactly opposite tendency. Thus the smallest number of eggs generally appears in the large first morning specimen, or in the immediately following specimen if this occurs soon afterwards. The small mid-day specimens usually contain the largest number of eggs.

If the worms produced eggs at a constant rate throughout the 24 hour period and if these eggs reached the lumen of the bladder at a constant rate, the number of eggs in each specimen should be positively correlated with the number of hours since the last specimen. The volume of urine would also be expected to show positive correlation with time. On the basis of these assumptions there should be a positive correlation between eggs per specimen and urine volume. The facts as seen from the graphs do not support this hypothesis.

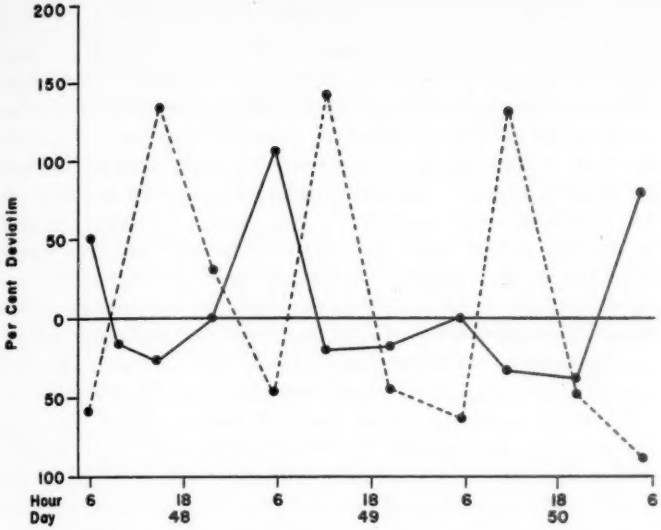


FIG. 2. Urine volume per specimen and eggs per specimen for three typical days of Case 1.

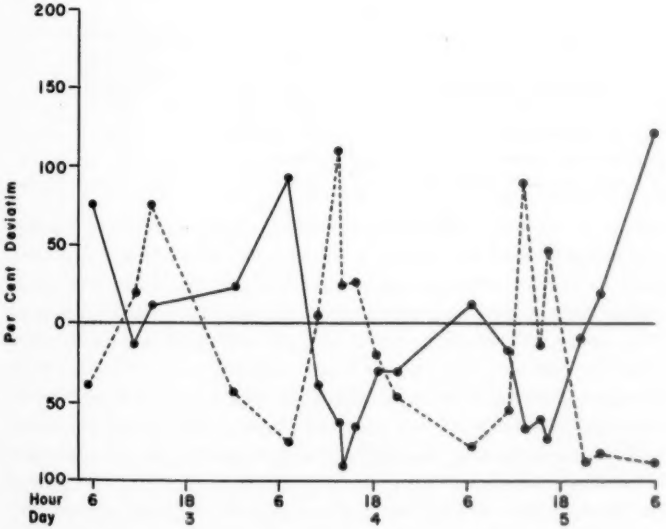


FIG. 3. Urine volume per specimen and eggs per specimen for three typical days of Case 2.

The most plausible hypothesis seems to be that some of the eggs are retained in the tissues during the quiescent sleeping period when the bladder wall is distending, with relatively few entering the lumen at this time. With contraction of the bladder some of the eggs are then extruded and passed in the first morning specimen. The individual's activity during the early part of the day with more frequent urination and contraction of the bladder might cause the eggs which were retained during the night to be extruded in relatively large numbers. This hypothesis would explain the high egg counts in the mid-day specimens. Once the reserve of eggs had been removed from the tissues by these activities, the number passed would then decrease toward the average number being produced by the worms.

To verify these conclusions the data were subjected to extensive analysis and reported in detail by Stimmel (*loc. cit.*). We will give only a brief summary here. Table 2 shows the mean values of the various factors concerned. Variation between speci-

TABLE 2

Mean values and their standard errors based on individual urine specimens for a 10 day period.

	CASE I	CASE II
Hours since last specimen.....	5.85 ± .63	4.55 ± .35
Urine volume in ml.....	330 ± 24	142 ± 9.0
Eggs per specimen.....	2,211 ± 228	9,528 ± 1,018
Eggs per ml. of urine.....	9.4 ± 1.3	108 ± 20

mens as related to the usefulness of single specimens in estimating the level of infection will be discussed later. The most fruitful analysis was by means of correlation and the analysis of variance in regression, the results of which are shown in table 3. The urine volume was shown to have a highly significant positive correlation with the number of hours since the previous specimen, as might be expected. The other zero-order coefficients indicate that there is a tendency for the number of eggs per specimen to be inversely related to the volume of the specimen and also to the length of time since the last specimen. The partial coefficients have the effect of showing how each of the two factors, volume and time, is separately related to the number of eggs

when the other factor is held constant. The fact that all of the partial coefficients showed lower values than the corresponding zero-order coefficients indicates that neither volume nor time is separately related to the number of eggs. The multiple correlation coefficients, however, indicate that taken together these two factors are inversely related to the number of eggs. The multiple regression equation for Case I was $Y = 3{,}475 - 65\,X - 2.68\,Z$, and that for Case II was $Y = 16.602 - 510\,X - 33.46Z$, where the symbols are those used in table 3. Although the multiple correlation coefficient for Case I is not indicated in the table as significant, the analysis of variance showed the multiple regression to be significant at about the 6 per cent level. That same factor for Case II was highly significant.

TABLE 3

*Correlation coefficients between the hours since the last specimen (x), eggs per specimen (y) and urine volume per specimen (z). Statistical significance of values is indicated by * for the 5 per cent level and by † for the 1 per cent level.*

	CASE I	CASE II
r_{xy}	—.28	—.35*
r_{yz}	—.35*	—.39†
r_{xz}	+.48†	+.54†
$r_{yx.z}$	—.13	—.14
$r_{yz.x}$	—.26	—.24
$R_{y.xz}$	+.36	+.41†

From a practical viewpoint this analysis means that there is good statistical support for the conclusions reached by inspection of figures 1 and 2. The daily cycle of egg output with a low number in the morning and increasing numbers until after midday and the opposite cycle of urine output are related to each other by more than chance, especially when time between specimens is taken into consideration. The point of greatest interest, which has been pointed out previously, is well substantiated by this analysis, namely that the long period between specimens during sleep results in large urine volume and small numbers of eggs. With increasing activity during the day and more frequent passage of small urine specimen's there is a resultant high egg output.

Evaluation of a Single Specimen as an Estimate

For clinical diagnosis of schistosomiasis and for estimating the prevalence of infection in epidemiological surveys a problem of great importance has been the differentiation of uninfected people from those infected people who are passing only the minimal number of eggs. Many publications have dealt with evaluation of various methods of diagnosing *Schistosoma mansoni* and *S. japonicum* under these conditions, the main problem being to find the eggs in the feces when they are scarce. In the case of *S. haematobium* it is relatively easy to find the eggs if they are present in the urine. The chief problem here is the type of case which passes so few eggs that they do not appear in every specimen of urine. Our data do not provide direct evidence on this point since no specimen in the series was without eggs. Indirectly it might be inferred from the diurnal cycle observed in these cases that, in cases passing very few eggs, the early morning specimen would be least likely to contain eggs. To this extent we can support the conclusion of Bennie (1949) that the commonly recommended practice of taking the early morning urine as the preferred specimen for diagnosis is fallacious. As far as we can judge from our cases the best specimen to take would be the first one passed after noon. In Case I this specimen contained the largest number of eggs passed during the 24 hour period on 6 days but not on the other 4 days. In Case II this specimen contained the largest number on all 10 days. Whether the same results would follow in cases passing fewer eggs is a matter of speculation.

Our data provide more satisfactory conclusions on the validity of using the number of eggs in a single specimen for estimation of the level of infection. Here we have the same difficulty of specifying which specimen should be taken. Since such counts will probably never have an absolute value but will only be used to estimate the relative egg output of different groups of people, the variability is more important than the mean count. On the other hand it would be inadvisable to use the first morning specimen since prevalence will also need to be determined simultaneously. The means and coefficients of variation of the egg content of various types of specimens are shown in table 4. The first morning specimen was chosen as the first one passed after arising and did not include the few passed shortly after midnight.

This specimen is not always the lowest in egg content but averages only slightly above the lowest. It has a relatively high coefficient of variation and from all viewpoints would be the poorest specimen to use. The specimen with the highest number of eggs not only gives the best estimate of prevalence but also has the lowest variability. It is of course impossible to specify in advance that such a specimen should be taken. The best approximation is the first afternoon specimen. In Case II this is always the specimen with the highest count. In Case I the mean of the first afternoon specimen is lower than that of the highest specimen and the variability is higher, but from either viewpoint it is better than a random specimen taken at an unspecified time.

TABLE 4

Means, standard errors and coefficients of variation of egg content for all specimens for the 10 day period, for the first morning specimen each day, for the first afternoon specimen, for the specimen with the lowest number of eggs, and for the specimen with the highest number of eggs.

	CASE I			CASE II		
	Mean	SE	CV	Mean	SE	CV
All specimens	2,211	±227	66	9,528	±1,018	80
Lowest specimen ...	712	±142	60	2,716	± 607	66
First morning	921	±171	56	3,170	± 687	65
First afternoon	3,031	±446	44	21,533	±2,457	34
Highest specimen ..	4,136	±360	26	21,533	±2,457	34

Discussion

No attempt has been made to thoroughly review the literature on the problem of making a negative diagnosis when no eggs are found on examination of a single urine specimen. Many authors have commented on the problem but few have published data. It seems obvious that the probability of finding eggs in a single specimen must be in some way proportional to the average number of eggs being passed over a period of time. It is to be hoped that someone will make a longitudinal study of cases passing relatively few eggs.

Part of this problem is the question of which specimen should be taken to insure the highest probability of finding eggs. We have not been able to locate the original source of the frequent

recommendation to take the last portion of the first morning specimen. Nor can we find any evidence supporting such a recommendation. As already stated, our data appears to substantiate the conclusion of Bennie (1949) that this is probably the poorest specimen to take. Our data indicate that the first specimen passed after noon would probably be the best for this purpose.

This problem of making a positive or negative diagnosis is of primary importance to the clinician. It is also important to the epidemiologist, but whereas the clinician deals with the individual case, the epidemiologist deals with groups of cases. The epidemiologist does not need to identify every positive case, but can collect data for the purpose of mathematically correcting deficiencies in the prevalence rates. The above mentioned longitudinal study of cases passing small numbers of eggs should establish such correction factors.

Our principal interest in the present study has been concerned with the problem of measuring the intensity of infection in cases known to be infected. Whether or not this can be done successfully by counting the eggs passed depends on further study of various aspects of the problem. There should be a clear understanding as to what is envisioned by those who advocate the procedure. No one who has studied such problems has advocated attempting to assess the level of intensity of individual infections except by prolonged study, which would be prohibitive in epidemiological work. The argument has been that on the average the intensity of infections in a group of persons, more or less homogenous as to age and sex, should be reflected in the average egg count. The opponents of such procedures have either failed to grasp this distinction between individuals and groups or have distrusted the statistical interpretations which must be placed on results of group studies. In spite of the many criticisms of the use of egg counts, the method has proved to be very fruitful in the case of intestinal nematodes, providing interpretations have been made with the proper caution.

The application of similar methods to schistosomiasis will involve certain difficulties not present in nematode infections. The principal factor is that the eggs are not laid directly in the lumen of an organ opening to the exterior and consequently fibrosis of the tissues in cases infected for a long time may interfere with

the passage of eggs. The first step in determining whether such methods will be feasible would appear to be a determination of the variability of egg output from specimen to specimen in the same individual.

Bennie (1949) studied a series of specimens from one case but was not interested in this aspect of the problem. Since she used only the last portion of each specimen, the data cannot be used to provide a measure of variability. Gerritsen *et al.* (1953) collected consecutive 12 hour specimens from 8 cases for about 10 days. When their data are converted to a 24 hour basis the coefficients of variation from day to day range from 52 to 112 per cent. Since the cases were subjected to unusual living conditions, it is not unexpected that the variability is greater than in our cases. Blackie (1932) considers the problem of using egg counts as a measure of intensity. The abstract of his paper by Clayton Lane in the Tropical Diseases Bulletin gives the impression that he looks with disfavor on the idea. Bennie (1949) quotes him out of context and gives a similar impression. Our impression from reading the original paper is quite different. He states, "It was frequently noted that daily specimens from the same individual showed considerable variation in the number of eggs present." He does not support this statement with data. He also states, "While it is realized that the number of schistosome eggs found in a given sample of urine cannot be regarded as an accurate index of the intensity of infection . . .," and then explains how he divided his cases into "light, moderate and heavy infections" by the ease of finding eggs in the specimen. He states further, "A general impression can be formed of the intensity of urinary infection from the method of recording the microscopical findings." These grades "refer only to the number of parasites present in the host," and are not to be taken as an "index of gravity of pathological changes." He uses these grades throughout his survey and clearly indicates his belief that the number of eggs passed do on the average reflect the number of worms present. By implication he also seems to infer that the pathological damage would on the average be correlated with the intensity.

In the many studies of intensity of nematode infections about two decades ago an attempt was made to correlate the egg output with the number of worms present and secondarily to correlate the number of worms with the clinical damage. It was found

later that the more direct correlation between egg output and clinical damage was more useful for comparative studies. Egg-worm correlations will be impossible in schistosomiasis, but the possibility of correlation of egg output with clinical damage should be explored. Before this can be done it will be necessary to show that the egg output can be adequately estimated on the average by examination of the amount of urine or feces which can be collected in a routine survey. On this latter point the present study provides some evidence.

In a routine survey about the best that can be expected is the collection of one entire urine specimen. Part of a specimen would be adequate if it were known to be representative of the whole. Further study needs to be made as to whether there are equal concentrations of eggs in the first and last parts of the passage. If an entire passage is requested without specifying at what time of day it should be passed, there will be a question of whether it would be a truly random one. Would there be a tendency in the population group under study to present a disproportionate number of specimens passed at one time of day? If the time is specified there will always be the question as to whether instructions were followed. Probably the most practical procedure would be to request any complete specimen and then attempt to obtain a record of the actual time of passage for at least a representative sample of the population. Some correction to the data could then be applied.

The coefficients of variation of eggs per specimen for truly random specimens for the two cases studied were 66 and 80 per cent. If these figures are used to establish confidence limits they appear unreasonably high. But if one considers the fact that estimates will not be made of individual infections, but only of the mean of groups, the indicated variability would be within reasonable limits and no higher than those successfully used in the study of nematode infections. If one could obtain the first afternoon specimen the variability would be cut in half, and even the first morning specimen would give some advantage over a random specimen except for the possible danger of missing light cases.

There is also the possibility of using eggs per milliliter of urine as the unit of measure rather than eggs per specimen. If a

specified quantity were to be taken from specimens representing only part of a passage, the problem of even distribution in the first and last portions would again arise. For determining prevalence Scott (1937) used eggs per 50 ml. of urine, which quantity was taken after stirring a specimen representing a complete passage. The data from the 10 day period of each of the two cases were studied from this point of view. The distribution by specimens of eggs per milliliter was extremely skewed, most specimens containing relatively few eggs. Coefficients of variation on this basis were 87 per cent for Case I, and 131 per cent for Case II. It is doubtful whether these coefficients based on skewed distributions should be directly compared with those mentioned previously, all of which were based on reasonably symmetrical distributions. Transformations showed the skewed distributions to be approximately log-normal with geometric means of 5.8 and 53.4 eggs per ml. respectively. Coefficients of variation calculated on the logarithmic scale were 63 and 32 per cent respectively. Converting to the basis of 50 eggs per ml. resulted in coefficients of variation on the logarithmic basis of 20 and 16 per cent respectively. In general the use of such coefficients based on transformed variables must be interpreted with care. Since distributions of eggs per 50 ml. in a cross section of the population were also found to be log-normal in the populations reported by Scott (1937), these latter coefficients of variation would seem to be applicable.

More studies of variability of output at various levels of intensity and with varying degrees of chronicity will certainly be needed before quantitative estimation can be attempted. In light of the present findings, however, the possibility does not look as hopeless as some have thought. As far as the variability of egg output is concerned a single specimen or even part of a specimen from enough people to represent a cross section of a population should be sufficient to estimate the average level of the output in the population. Other studies will be needed to show that such an estimate is correlated with the amount of damage being done by the infections.

BIBLIOGRAPHY

Barlow, C. H.: A New Method for Examining Urine for Helminth Eggs, *Am. J. Hyg.*, 14: 212–217, 1931.

Barlow, C. H.: A Theory of Egg-Deposition by *Bilharzia haematobia*. *J. Parasit.*, 35: 205–207, 1949.

Bennie, Iris: Urinary Schistosomiasis. The Best Time to Obtain Specimens. The Effect of Specific Therapy on Egg Output, *S. Af. Med. J.*, 23: 97–100, 1949.

Blackie, W. K.: A Helminthological Survey of Southern Rhodesia, *Lond. Sch. Trop. Med. and Hyg. Mem.*, Series 5, 91 pp., 1932.

Brumpt, E.: La Ponte des Schistosomes, *Ann. de Parasit. Hum. et Comp.* 8: 263–297, 1930.

Gerritsen, T., A. R. P. Walker, B. de Meillon, and R. M. Yeo: Long Term Investigation of Blood Loss and Egg Load in Urinary Schistosomiasis in the African Bantu, *Trans. Roy. Soc. Trop. Med. and Hyg.*, 47: 134–140, 1953.

Kohlschütter, E., and Enrique Koppisch: On the Mode of Extrusion of Schistosome Ova from Blood Vessels into the Tissues, *Schweiz. Ztschr. Path. U. Bakteriol.*, 4: 357–369, 1941.

Scott, J. Allen: The Incidence and Distribution of the Human Schistosomes in Egypt, *Am. J. Hyg.*, 25: 566–614, 1937.

Scott, J. Allen: The Regularity of Egg Output of Helminth Infestations, with Special Reference to *Schistosoma mansoni*, *Am. J. Hyg.*, 27: 155–175, 1938.

Stimmel, Carolyn M.: A Statistical Study of Helminth Egg Output. Thesis, University of Texas Medical Branch Library, 47 pp., 1956.

van den Berghe, L.: Quelques Faits Nouveaux Concernant La Migration et La Ponte des Schistosomes, *Ann. de Parasit.*, 15: 354–363, 1937.

EFFECTS OF THE BLOOD DERIVATIVE, SANGUININ, ON PLANT TISSUE (LUPINUS ALBUS)*

DOROTHY M. WHITNEY AND MARIO PAVAN

The biological activities of unpurified Sanguinin, a bovine blood derivative, have been demonstrated by its stimulating effect on granulation tissue in wounds of man and animals (Nilasena *et al.*, 1954; Mullins *et al.*, 1954; Bader, 1952) and its inhibiting effect on bacterial growth (Anigstein *et al.*, 1950; Whitney *et al.*, 1950). It was of interest to investigate whether a cellular response from growing plant tissue could be expected, inasmuch as one of us (M. Pavan) had demonstrated marked inhibitory effects on growth of roots of *Lupinus albus* placed in contact with iridomyrmecine (Pavan, 1955), a biologically active product of animal origin.

Materials and Methods

Sanguinin, a lyophilized product derived from bovine blood by proteolytic enzyme hydrolysis, was prepared at the University of Texas Medical Branch (Whitney et al., 1950). The standard Macht test (Macht, 1941) used to evaluate effects of various pharmacological compounds on the development of germinating seedlings was adapted to the study of Sanguinin. Seeds of *Lupinus albus*, large Italian variety, soaked in water 24 hours, were germinated in sand 2 or 3 days until the roots reached 30 to 40 mm. in length. They were then divided into 4 groups of 15 seedlings each, and initial measurements recorded. The roots of one group of seedlings were immersed in Shive nutrient solution (Macht, 1941), to which 1 mg. Sanguinin/ml. was added, each seedling in a separate tube. A second group of seedlings was treated in the same manner with 0.1 mg. Sanguinin/ml. A third group of germinating seeds was similarly grown in tubes containing 0.05 mg. Sanguinin/ml. Shive medium. A fourth group was placed in tubes containing only the nutrient medium. All 4 groups were allowed to develop in the dark at 20° C. and at

* From the Department of Preventive Medicine and Public Health, University of Texas Medical Branch, Galveston, Texas, and Istituto di Anatomia Comparata, del' Universita di Pavia, Pavia, Italy.

Supported by a faculty fellowship from the James W. McLaughlin Fund of the University of Texas Medical Branch. Thanks are due to Dottoresse Albertina Baggini, Mara Miradoli, Maria Louisa Valcurone, and Signorina Maria Teresa Vigani for technical assistance. Credit is given to Dr. J. Allen Scott, Professor of Epidemiology and Medical Statistics and Miss Carolyn Stimmel, University of Texas Medical Branch for statistical analysis. Received for publication July 29, 1956.

1, 2, and 5 days, observations were made and measurements of the roots were recorded. At 2, 5 and 9 days direct silhouette prints were made on photosensitive paper of 3 developing seedlings chosen at random from each group of 15.

Results

Growth of roots of *Lupinus albus* in Shive medium (containing 1.0 mg. Sanguinin/ml.) was inhibited significantly by 1.0mg./ Sanguinin just as shown by statistical analysis (Table 1). Begin-

TABLE 1

THE MEAN AND THE STANDARD ERROR OF THE LENGTH OF THE ROOTS OF LUPINUS ALBUS IN MILLIMETERS, GROWING IN SHIVE MEDIUM WITH VARYING AMOUNT OF SANGUININ, 15 SEEDS TO A GROUP AT 1, 2, AND 5 DAY INTERVALS.

SANGUININ PER ML SHIVE	INITIAL mm	1 DAY mm	2 DAY mm	5 DAY mm
0	37.4 ± 0.4	70.5 ± 1.4	90.9 ± 2.0	136.0 ± 3.5
1.0 MG.	38.0 ± 0.4	63.1 ± 1.1	75.3 ± 1.8	118.0 ± 1.9
T*		4.4**	5.9**	4.8**
0.1 MG.	37.5 ± 0.5	72.3 ± 1.3	97.0 ± 1.9	157.3 ± 3.5
T		-1.0	-4.2**	-4.3**
0.05 MG.	36.9 ± 0.6	70.3 ± 1.8	93.4 ± 2.6	150.3 ± 4.5
T		0.12	-0.7	-2.4*

*T VALUES FOR DIFFERENCES BETWEEN MEANS OF CONTROLS AND MEAN OF EACH TREATED GROUP.

*SIGNIFICANT AT 0.05 LEVEL OF PROBABILITY.
**HIGHLY SIGNIFICANT AT 0.01 LEVEL OF PROBABILITY.

ning with first day of observation, the mean, 63.1 mm. of the Sanguinin treated group was considerably lower than the mean of controls measuring 70.5 mm. On the second day the mean root length of the Sanguinin treated group was 75.3 mm. as compared with 90.9 mm. in the controls. Roots became deep brown and showed a marked increase in diameter with lateral splitting of outer cell layers. In 5 days, the mean of the Sanguinin treated group was 118 mm., that of controls, 136 mm. This stunting of growth was highly significant by the "t" value and the corresponding P value, which was 0.01.

Obversely, when the concentration of Sanguinin was reduced to 0.1 mg./ml. Shive nutrient medium, a stimulating effect on root growth was found. While growth acceleration was noted on

the first day, the differences in lengths of the treated and untreated roots became statistically highly significant on the second day (97 mm. as compared with 90.9 mm. of the controls). On the fifth day, root growth showed a highly significant acceleration in the Sanguinin treated group with a mean of 157.3 mm. as compared with the control mean of 136.0 mm.

Acceleration of growth of the roots was also noted in the series of developing seedlings treated with 0.05 mg. Sanguinin/ml. medium, but to a smaller degree than those treated with 0.1 mg. Sanguinin/ml.

Tables 2 and 3 show graphically the development of roots of *Lupinus albus* in concentrations of Sanguinin used, the inhibitory

Table 2

Growth curves in mm of the roots of Lupinus albus in varying concentrations of Sanguinin in Shive medium, based on the mean root length of 15 beans to a group at 1, 2 and 5 day intervals.

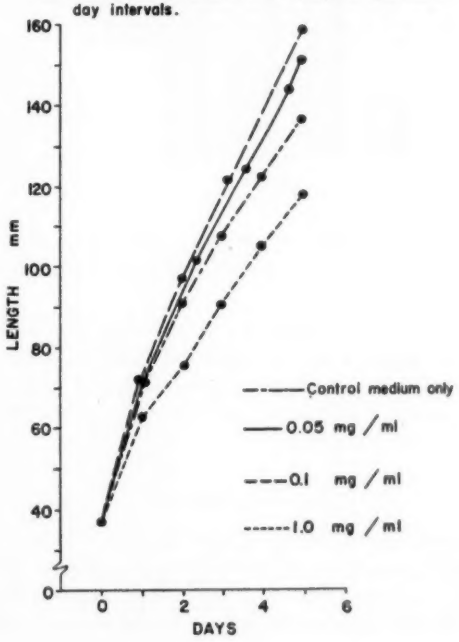
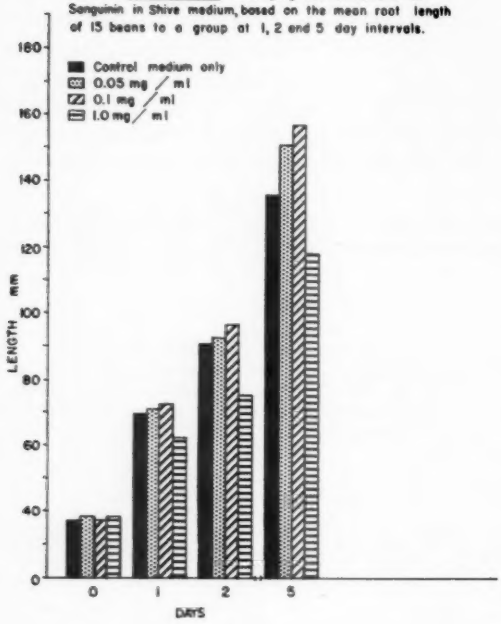


Table 3

Graphic presentation of the growth in mm of the roots of Lupinus albus in varying concentrations of Sanguinin in Shive medium, based on the mean root length of 15 beans to a group at 1, 2 and 5 day intervals.



effect of 1.0 mg. Sanguinin/ml., the stimulatory effect of 0.1 mg. Sanguinin/ml., and the weaker stimulatory action of 0.05 mg./ml. concentration.

Although both inhibitory and stimulatory effects of Sanguinin on the developing roots were highly significant, it was also noted that in all concentrations, the lateral roots were more abundant, and the foliation more advanced at the 9 day period than seedlings not treated with Sanguinin.

Summary

Lupinus albus seedlings under standard conditions of the Macht test, when kept in various concentrations of unpurified Sanguinin (1.0, 0.1, 0.05 mg./ml. Shive nutrient solution) exhibit significant cellular responses. In the highest concentration

used, the roots were inhibited in longitudinal growth and at the same time were increased in diameter. On the other hand, the lower concentrations of Sanguinin exhibited marked stimulatory effects on roots of developing seedlings. Enhancement of growth of lateral rootlets and foliage was noted in all concentrations at the end of 9 days.

BIBLIOGRAPHY

Anigstein, L., D. M. Whitney, and Don Micks: Antibacterial Factor in Tick Extracts, *Texas Rept. on Biol. and Med.*, 8: 86–100, 1950.

Bader, M. N.: Wound-healing Properties of Blood Hydrolysate, Sanguinin, *Texas Rept. on Biol. and Med.*, 10: 773–777, 1952.

Macht, David I., and Moses B. Macht: Phytotoxic Reactions of Some Blood Sera, *Jour. of Lab. and Clin. Med.*, 26: 597–615, 1941.

Mullins, J. Fred, E. S. Farrington, and C. J. Wilson: Sanguinin in Ischemic Ulcers, *Texas State Jour. of Med.*, 51: 507–508, 1955.

Nilasena, Samchai, Fenwick L. Watts, J. Fred Mullins, and Ludwik Anigstein: Sanguinin: Local Wound Healing Effect on Chronic Cutaneous Ulcerations, *Texas Rept. on Biol. and Med.*, 12: 1047–1051, 1954.

Pavan, M.: Gli insetti come fonte di Prodotti biologicamente attivi, "*La Chimica e l'Industria*," 77: 714, 1955.

Whitney, D. M., L. Anigstein, and Don Micks: Antibacterial Activity of Hydrolysed Red Blood Cells in Vitro, *Proc. Soc. Exp. Biol. and Med.*, 74: 346–350, 1950.

EFFECT OF CERTAIN DYES ON GROWTH *IN VITRO* OF *ENDAMOEBA HISTOLYTICA*

ATHANASIUS EUL JOON YOO* AND G. A. EMERSON

Dyes have been of prime interest in experimental chemotherapy ever since they were studied systematically almost 50 years ago in experimental trypanosomiases by Ehrlich (1907); the first effective sulfonamide was later discovered through the activity of its azo dye. Further interest in dyes arises from the lore accumulated in Chinese medicine over some 4,000 years of clinical experience contributing to theories of action of colored agents, based on biotrepologic concepts of specifically favorable colors for individual organs of the body affected by disease.

In vitro study of susceptibility of pathogens to chemical agents does not invariably result in information of value in developing new chemotherapeutic agents. Generally good correlation exists between *in vitro* amebacidal actions and clinical effectiveness of representative agents now used in amebic dysentery, even with full appreciation that certain agents in either instance may exert their major effect against symbiotic bacteria rather than directly against the pathogen. In contrast, *in vitro* estimations of fungicidal activity may be of little value since forms of certain pathogenic fungi in tissues differ morphologically, and probably functionally, from those grown *in vitro* (Dennis *et al.*, 1955).

Toxicity to the patient of amebacides active *in vitro* is a deciding factor in determining their clinical usefulness (Rinehart and Anderson, 1931). The tremendous variation in toxicity of different dyes to man has frequently been forgotten as a consideration in their relative usefulness as chemotherapeutic agents, exemplified in experimental clinical therapy of a mycobacterial infection (Ryrie, 1933).

---

* From the Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston. Presented to the Southwestern Section, Society for Experimental Biology and Medicine, Oklahoma City, November 18, 1955. Received for publication August 29, 1956. Lederle Fellow, August, 1955, and James W. McLaughlin Fellow September 1, 1955–August 31, 1956. University of Texas Medical Branch, from Holy Ghost Medical College, Seoul, Republic of Korea.

Certain dyes have previously been tested as amebacides. Conn (1946) notes that brilliant cresyl blue is an amebacide; in the present study, 0.1% of this dye did not inhibit growth of *Endamoeba histolytica* in a liquid egg-yolk-liver medium. Sah (1950) notes amebastatic concentrations in a similar medium of 5 bisazo dyes derived from tetrazotized bis(4-aminophenyl) sulfone and, respectively, 3,5-diaminobenzoic acid, as 0.1%; 2-methyl-1,4-naphthohydroquinone, as 0.1%; *p*-aminobenzoic acid, as 0.2%; phloroglucinol, as about 0.2%; and 8-amino-1-naphthol-3,6-disulfonic acid, as >0.2%.

For screening purposes, and to further both biochemorphic and biotrepologic knowledge in this field, tests were made of the *in vitro* activity of 21 dyes against vegetative *E. histolytica* grown in Balamuth's medium (Balamuth, 1946).

Materials

In clinical material, as usually isolated, gas-forming bacterial contaminants confuse results of estimations of amebacidal effects of agents in diphasic media such as Endamoeba Medium (Difco) used according to recommendations of Cleveland and Collier (1930). Such cultures, isolated by Miss Alberdena Ruysenaars of the Department of Bacteriology and Parasitology, University of Texas Medical Branch, could not be freed of gas-forming bacteria by incorporation of various antibiotics in the medium.

Dr. Robert Yeager, of Tulane University, generously supplied us with a strain of *E. histolytica* which grows well in a modified Balamuth's medium, accompanied only by slight growth of a small bacillus. Since factors of precision in preparing exact concentrations of agents are greatly simplified by using liquid rather than diphasic media, Balamuth's medium is superior for screening-tests of amebacides, regardless of the presence or absence of gas-forming organisms.

A modified Balamuth's medium used in all of the present study was prepared according to suggestions of Dr. Yeager, as follows:

Thirty-two fresh eggs are placed in boiling water or flowing steam for 15 minutes. Their yolks are homogenized with 1000 ml. of 0.8% NaCl solution in a Waring Blendor, and heated at 80° C. for 20 minutes, with frequent stirring. To the cooled suspension, 160 ml. of water are added, and the bulk of the pulp is separated from the aqueous phase by squeezing the mixture through a thick cotton towel. The volume of the turbid, yellow, aqueous phase is made up to 1000 ml. by addition of 0.8% NaCl solution. This crude extract

is then placed in two 1 L. flasks, autoclaved at 121° C. for 20 minutes, and allowed to remain overnight at 3° C. It is then filtered through 2 pieces of Whatman No. 3 paper, with moderate vacuum; only small amounts are added to the filter at any time, and the filter papers are replaced frequently, as indicated by the nature of the filtrate, in order to ensure a clear filtrate. An equal volume of *M*/15 phosphate buffer of pH 7.5 and 5.0 ml/L. of Crude Liver Extract No. 408 (Eli Lilly and Co., Indianapolis) are added to the filtrate. The medium is dispensed in 4.8 or 9.6 ml. aliquots in test-tubes, and autoclaved at 121° C. for 20 minutes. Approximately 400 tubes containing 4.8 ml. of medium may be prepared from 32 eggs.

Tubes of this medium are kept at 3° C. until used, at which time a large loopful of sterile rice powder (ca. 10 mg.) is added aseptically. In preliminary tests with diphasic media, a sterile, freshly ground rice powder proved more stimulant to growth than a commercial preparation of rice powder, and so was used for all of the present study.

The 21 dyes used were obtained from various commercial sources, and most were certified by Dr. H. J. Conn, of the Biological Stain Commission. Concentrations used in all of the present studies were adjusted to conform to the declared dye content of each sample of dye.

Methods

Before addition to the sterile medium and immediately before inoculation, dyes were dissolved in 0.85% NaCl solution and filtered through a sterile Morton filter (Morton, 1944). Further dilution of the filtered dye solutions was made with sterile 0.85% NaCl solutions to permit exact measurement of final concentrations of dye in the medium, over the range of 10^{-3} to 10^{-6}.

Inocula were prepared from cultures in Balamuth's medium after 48 hours of growth, with previous subcultures made each 48 hours by transfer of 0.2–0.4 ml. of sediment from the bottom of tubes of cultures grown for 48 hours. Inocula into the dilutions of dyes in the medium were also 0.2/4.8 ml. or 0.4/9.6 ml.

Sediment from each experimental culture tube was examined microscopically after 48 hours of incubation at 37 ± 2° C. for numbers of *E. histolytica* present and their motility. Absence of motile organisms was taken as the criterion of the inhibitory effect of the dye tested.

Results

Of the 21 dyes tested, four were effective at a concentration of 0.01% but not at 0.001%. These dyes were naphthol green (naphthol green B, green PL, acid green O), auramine (auramine

O, canary yellow, pyoktaninum aureum, pyoktanin yellow), pararosaniline (magenta O, basic rubin, parafuchsin, paramagenta), and rose bengal (rose bengal extra, 3B conc., N extra, DY, B and 2B). These are, respectively, nitroso, diphenylmethane, triamino-triphenylmethane and fluoran dyes.

Eight dyes were active at a concentration of 0.1% but not at 0.01%. These were gentian violet (a poorly defined mixture of violet rosanilines), methyl blue (cotton blue, helvetia blue), iodophthalein (tetraiodophenolphthalein), azure A (dimethylthionin), Nile blue sulfate (Nile blue A), celestine blue (celestine blue B, coreine 2R), toluidine blue (toluidine blue O, methylene blue T 50, methylene blue T extra) and toluylene blue (a homologue of Bindschedler's green). These dyes represent a heterogeneity of chemical structures.

Nine dyes were ineffective at a concentration of 0.1%. These were malachite green (Victoria green B, Victoria green WB, new Victoria green extra, O, I, II, diamond green B, BX, P extra, solid green O, light green N), ethyl violet (ethyl purple 6B), methyl orange (orange II, helianthin, gold orange MP, tropaeolin D), Magdala red (naphthalene red, naphthalene pink, naphthylamine pink, Sudan red), xylene cyanole FF (cyanol FF), azure C (monomethylthionin), Niagara sky blue (pontamine sky blue 6BX, Niagara sky blue 6B, direct sky blue), brilliant cresyl blue (cresyl blue 2RN, cresyl blue BBS, brilliant blue C) and Evans' blue (a homologue of trypan blue, differing in position of sulfonate groups). These agents also represent a heterogeneous group of chemical structures.

Discussion

In the concentrations used, none of the dyes significantly affects the pH or causes other gross physicochemical changes in the medium other than its color. Finer, undetermined physicochemical effects may well be due to specific removal of trace nutrients, but such effects may equally be considered reasonably as direct effects upon components of the pathogen.

Activities of the dyes tested show no gross biochemorphic relations. Absorption of visible light at specific wavelengths is a fastidious index of electron distribution in organic compounds and so of finer structural relations than may be apparent grossly

from structural formulae (Henrich, 1922; Brode, 1955). However, chromogenicity and other attributes of color are unrelated to the amebacidal activities of the dyes studied.

Toxicity of possibly effective doses of the dyes studied probably precludes serious consideration of even the more effective dyes for clinical trial in amebic dysentery. Rose bengal, for example, might possibly be useful clinically if concentrations above 10 mg. % could be obtained in all loci containing amebae; the maximum intravenous dose of this agent considered safe for liver-function tests is only 3 mg./kg. (Sollmann and Hanzlik, 1939). This dose corresponds to a concentration of 0.3 mg. %, and toxic symptoms have occurred even with this dose.

Summary

Twenty-one dyes were tested in Balamuth's medium for inhibition of growth of *E. histolytica*, in concentrations of 10^{-3} to 10^{-6}. Of these dyes, naphthol green, auramine, pararosaniline and rose bengal are effective in concentrations of 0.01% but not 0.001%. Gentian violet, methyl blue, iodophthalein, azure A, Nile blue sulfate, celestine blue, toluidine blue and toluylene blue are effective at 0.1% but not 0.01%. Malachite green, ethyl violet, methyl orange, Magdala red, xylene cyanole FF, azure C, Niagara sky blue, brilliant cresyl blue and Evans' blue are ineffective at 0.1%.

REFERENCES

Balamuth, W.: Improved egg yolk infusion for cultivation of *Entamoeba histolytica* and other intestinal protozoa, *Amer. Jour. Clin. Pathol.*, 16: 380–384, 1946.

Brode, W. R.: Color and chemical constitution, *Amer. Scientist*, 43: 259–284, 1955.

Cleveland, L. R., and J. Collier: Various improvements in the cultivation of *Entamoeba histolytica*, *Amer. Jour. Hyg.*, 12: 606–613, 1930.

Conn, H. J.: *Biological Stains*, Monograph on Brilliant Cresyl Blue, p. 99, Biotech Publications, Geneva, N. Y., 1946.

Ehrlich, P.: Chemotherapeutische Trypanosomen-Studien, *Berlin. klin. Wochenschr.*, 44: 233–236; 280–283; 310–314; 341–344, 1907.

Dennis, James L., and A. E. Hansen: Coccidioidomycosis in children, *Pediatrics*, 14: 481–494, 1954.

Henrich, F.: *Theories of Organic Chemistry* (transl. by T. B. Johnson and D. A. Hahn), chapter 16, The relationship between color and chemical constitution, pp. 382–478. John Wiley and Sons, N. Y., 1922.

Morton, H. E.: A new style assembly for fritted filters, *Jour. Bact.*, 47: 379–380, 1944.

Rinehart, J. F., and H. H. Anderson: Effect of emetine on cardiac muscle, *Arch. Pathol.*, 11: 546–553, 1931.

Ryrie, G. A.: A preliminary report on the action of certain dyes in leprosy, *Trans. Royal Soc. Trop. Med. and Hygiene*, 27: 85–92, 1933.

Sah, P. P. T.: The antitubercular activity of azo dyes. I. Bisazo dyes derived from tetrazotized bis(4-aminophenyl)-sulfone and various coupling agents, *Recueil des Travaux Chimiques Pays-Bas*, 69: 1407–1434, 1950.

Sollmann, T. H., and P. J. Hanzlik: *Fundamentals of Experimental Pharmacology*, pp. 307. J. W. Stacey, Inc., San Francisco, 1939.

TENTH ANNUAL SYMPOSIUM ON FUNDAMENTAL CANCER RESEARCH

The University of Texas
M. D. Anderson Hospital and Tumor Institute

JOSEPH C. AUB
1956 Bertner Award Recipient

INTRODUCTION

R. Lee Clark, Jr.
Director and Surgeon-in-Chief

Research in biology, biochemistry, pathology and related fields may eventually answer the questions of what abnormal growth is, how it comes about, and how it is to be controlled. For this reason, The University of Texas M. D. Anderson Hospital and Tumor Institute inaugurated an annual symposium on fundamental cancer research in 1946. The aim was to have speakers of outstanding research reputation take part in the meeting and thus establish and maintain a gathering where important current research would be reported, discussed and evaluated.

This year, for the first time, the first day of the symposium was devoted to reviews by the principle investigators of fifty-two research projects currently under investigation at The University of Texas M. D. Anderson Hospital and Tumor Institute. In order that the day be of more value than simple reporting, a research consultant panel was invited to question and discuss the projects. Members of the committee were: Dr. Murray Copeland, chairman of the Cancer Control Committee, National Cancer Institute; Dr. J. Murray Luck, President of the American Society of Biological Chemists; Dr. Theophilus S. Painter, Distinguished Professor of Zoology, The University of Texas; Dr. Frederick S. Philips, Research Consultant, American Cancer Society; Dr. C. P. Rhoads, Director of Sloan-Kettering Institute and member of the National Research Council; Dr. C. W. Shilling, of the Division of Biology and Medicine, Atomic Energy Commission. This committee gave constructive suggestions on many of the projects. In the future, it will be most interesting to follow the Annual Research Progress Reports, which hereafter will be presented on the first day of the Symposium.

The high point of the Symposium is the presentation of the Annual Bertner Foundation Award and delivery of the Bertner Lecture. Established in 1950, to honor the first acting director of M. D. Anderson Hospital and the first president of the Texas

Medical Center, the late Dr. E. W. Bertner, the award is presented in the form of a medallion to a person who the Awards Committee feels has made an outstanding contribution to the field of cancer research.

Recipient of the Sixth Bertner Foundation Award was Dr. Joseph C. Aub, professor of Research Medicine, Harvard Medical School and Director of Medical Laboratories at the Collis P. Huntington Memorial Hospital, Massachusetts General Hospital, Boston, Massachusetts. Dr. Aub, in over 40 years of active research, has felt that his greatest responsibility was to his young associates, and in encouraging them, he could thus provide for a continuity of research for the complex field of cancer study. Of no less importance are the scientific contributions he has made, such as the studies of metabolic physiology, in which he established the methods which are the foundation for present day metabolic studies—studies which are being recognized for their increasing importance toward a true understanding of cancer.

In this issue of *Texas Reports on Biology and Medicine*, Part I of the Symposium papers will appear, accompanied by the Bertner Lecture. Part II of the Symposium, Nucleic Acid and Metabolism, will appear in the spring issue of the journal.

Each year, the complete papers of the Symposium have been published in *Texas Reports on Biology and Medicine*, through the courtesy and cooperation of the editor. We wish particularly to thank Dr. John G. Sinclair, editor of the journal, for his splendid cooperation in carrying on the tradition of having the papers presented at this Texas meeting appear in this well-recognized Texas journal.

CANCER RESEARCH IS GROWING UP

JOSEPH C. AUB, M.D.*

We are all gathered here tonight really to honor the well known surgeon Dr. Ernst Bertner, for this award was named after him because of his remarkably active and successful medical life. He was always interested in cancer and he served as acting director at the M. D. Anderson Hospital during its formative first four years. He had been a vice president of the American Cancer Society and shortly before his death received an American Cancer Society award for distinguished service in cancer research. He died a victim of the disease to which he had given so much of his life. He also taught and was professor and chairman of the Department of Gynecology at Baylor University College of Medicine. His 61 years were filled with the activities and good deeds of an over-busy surgeon and, most important, I am told by those who knew him, filled with the amenities of good friendship.

It is a great pleasure of course to accept the Bertner award, particularly for me this year because my retirement from the University is imminent. Therefore, it has occurred to me that this is a suitable time to review laboratory research in cancer from 1929 when I started, to the present time. In 1929, cancer research was dominated by pathologists, and the meetings of the Cancer Research Society were largely occupied with the pathological appearance of tumor cells. Cancer research was for the most part done with transplantable tumors, except for the production of animals' own tumors by tar. In 1930 a new field was opened by the lovely purification and isolation of carcinogenic agents by Kennaway and Cook. Since then it has been easy to make animals produce tumors from their own body cells. The biochemical approach had been opened by the interesting work of Warburg in 1925.

My preparation for studying cancer was a good many years of physiological and chemical investigation of the internal secre-

* Medical Laboratories of the Collis P. Huntington Hospital of Harvard University at the Massachusetts General Hospital, Boston. This is publication No. 884 of the Cancer Commission of Harvard University.

tions, followed by studies of lead poisoning, which led to our pioneering in calcium metabolism. It was natural that I should want to use my physiological training, and our first laboratory activities were to try to control liver regeneration in the rat. These experiments were undisciplined, and they quickly taught us that liver regeneration was very complicated and compulsive. In 1930, I presented the problem of our future orientation to the Harvard Cancer Commission. We could probably write many papers if we devoted our time to techniques for producing tumors, or we might be quite unproductive but have a chance to really make fundamental progress if we studied normal and pathological growth and regeneration. Nothing really being known about the control of growth, we wanted to attack the fundamental problems and try to influence normal and abnormal growth and to learn the fundamental influences involved. The Harvard Cancer Commission was composed of men of good and considerable judgment. After a lengthy discussion, they agreed that the study of growth was the fundamental approach, and, therefore, the wiser one. Since that time our laboratory has worked on such problems.

While I feel certain that the right decision was made at that time, for the following fifteen years it was not generally considered so by influential workers in the country. It has only been in the last ten years that normal and abnormal growth has been generally considered a suitable subject for fundamental cancer investigations. There are two ways of approaching the cancer problem. One is at the bottom of the pyramid where fundamental knowledge should be sought. The other approach is at the top of the pyramid of knowledge of a disease. In this area, the control of the disease and efficient treatment are the goals. Our laboratory works at both levels now, but the greater stress is devoted to the fundamental approach in a rather typical way. In my lifetime, one gets closer and closer to the subject at hand. First, we were very remote, having to be content to study urine and stools or respiratory exchange. Then new techniques came in for analyzing the blood. But this, too, was remote from fundamental abnormalities. Now, in the last ten years, we have been learning rapidly how to dissect the cell chemically, to see what parts of the cell are involved in different metabolic reactions, and, in this way, to see how the reactions of cells of one organ differ from

those of another. It takes energy to divide, hence it is important to know how and where the energy is derived in cells which are dividing as often as cancer cells. Of course, the whole country is in full cry in this search for the cause and cure of cancer. It would be manifestly impossible to cover this field in a short talk. I cannot even do justice to the groups in our laboratory. But it is their work which I would like to describe briefly for you now.

First, normal regeneration—one of our perpetual problems, and this is now Dr. Nancy Bucher's pet project. Some years ago, she made a beautiful observation. She sewed three sibling rats together. After three or four months she then removed ⅔ of the livers of the two outside partners. The control amount of cell division could be determined by counting the two partial livers removed. The central rat was intact except that its blood was mixed with the other two and this apparently produced a very high rate of cell division in the liver of the intact animal. The rate of cell division of other organs was not higher than normal, showing the extraordinary specificity of this cellular function. Obviously something carried in the blood stream made these liver cells divide—but all the experiments which she has done subsequently have failed to demonstrate the factor. Thus prolonged transfusion with blood from hepatectomized rats or depletion of plasma proteins, for example, produce no increase of cell division.

She then turned to cholesterol synthesis in the liver as a synthetic activity possibly related to cell division. It was known that this could be made from radioactive citrate by slices of liver tissues. But this had never been produced by homogenates, preparations of broken-up cells, in which no intact cell remained. She promptly accomplished this by using a poorly fitting tool, and this allowed the study of what parts of the cell are involved in the synthesis. Now she has demonstrated that cholesterol is made by two factors in the normal cellular cytoplasm—the microsomes and the structureless fluid. It is not too much to expect that the mechanisms involved in this synthesis will soon be well elucidated by her and other workers in the country.

Dr. Paul Zamecnik, has a very distinguished group of young scientists around him, and they are dedicated to the problem of how proteins are synthesized in body cells. Their hope is that a clearer understanding of this fundamental problem will provide

insight into the rapid, poorly regulated rates of protein synthesis of neoplastic cells. These extraordinarily complicated and specific compounds can now also be made in homogenates, and the parts of the cell can be separated one from the other by varying speeds of centrifugation. Thus the areas in the cell which are used for synthesizing can be determined. The new proteins are recognized by using tagged amino acids. These building blocks of the proteins are made by Dr. Loftfield with radioactive carbon. The tagged amino acid can be recognized in the finished protein.

During the past year this group has learned much of the source of energy and the mechanism of uniting amino acids into proteins. How amino acids are lined up in order is indeed a problem.

All such metabolic transformations and also cell division and growth require energy, much of which comes from high energy phosphorus compounds. Doctors Scott and Taft have developed handsome micro techniques for analyses for these compounds in such a way that the amounts of different phosphorus compounds can be determined per cell. With rat livers prepared by butter yellow, one can progress from nearly normal through cirrhotic to hepatoma cells in serial sections. Their methods are so designed that frozen serial sections of tissues can be used in which the first is for microscopic characterization, and the next four or five sections are chemically analyzed. Thus quantitative results on a known number of cells become available. The ratio of acid soluble phosphorus compounds and of ribonucleic acid appears to be different in cancer than in the other liver constituents which they have measured. The importance of this abnormality is not clear.

The activities already described deal with chemical dissection of the cells, and the last decade will probably be remembered because of this type of research, the decade when cells began to fall apart chemically, and when the marvelous intracellular enzyme reactions began to be understood.

But we have not limited work to this activity. There is also the exciting fact that the internal secretions change cells in ways unknown. The vast amount of clinical investigation by Dr. Ira Nathanson and others showed that many cancers of the breast respond to sex hormones by improvement or acceleration. Their effect on tumor growth is largely unpredictable, and may be dramatic. Therefore, it is important to know where various hor-

mones are produced and how they are metabolized in the body. Dr. Lewis Engel has devised new methods which allow this to be done, and, with radioactive tagging of sex hormones, his workers can follow hormones in the body and determine the patterns of their breakdown. This important subject was also studied by Dr. Dobriner. Unfortunately, this handsome work has not demonstrated a pattern unique for people with cancer, but the work is not yet complete. One would expect some generalization to emerge, for there is such a difference in various cancers of the breast, some of which respond well to change of sex hormone balance in the body—while others progress relentlessly without regard to hormones. The source of various compounds and the various transformations in the body are becoming obvious. Recently, with Dr. Billy Baggett, it has become clear that the male hormone may be changed to female ones by exposing them to slices of the ovary or testis or adrenal. How fruitfully complicated this makes the problem.

As Dr. Zamecnik suggests, the story of Adam's rib appears in reality a biochemical prophecy. The same tiny synthetic particles of the cell—microsome—which have been found in Dr. Zamecnik's and Dr. Bucher's laboratories to be involved in protein and cholesterol synthesis, also now appear to be essential in the oxidation of the sterol side chain. We now begin to witness a unifying simplification, in this development of understanding of the growth machinery of the cell. The mitochondrion serves as the cell powerhouse, and the microsome emerges as the site where syntheses are carried out. The biochemist has a tendency to throw away the cell membrane, in distaste for its refusal to permit access of his substrates to vital cell centers. Surely it deserves attention for its possible role in the poorly regulated growth of the tumor cell. Therefore, I plan to devote much of my remaining years to the role of the cell surface in metabolic processes.

The work on hormones must be related to human tumors; and a close collaboration exists between the laboratories and our large clinical research group. As the internal secretions are clinically altered in patients, the chemical pattern of their excretion rates is carefully studied in Dr. Engel's laboratory. Our bright young clinicians also try to fathom the mechanisms by which chemotherapeutic agents exert their specialized actions on only certain tumors and do not influence other types of tumors. This involves

careful work on the metabolic research ward. Not only the obvious influence on the tumors themselves, but also the effects on the host must be studied, judged roughly by calcium and nitrogen balances, and the like. We evaluate the effectiveness of these agents and attempt to determine their mode of action both on the tumor cells and host cells. Therefore, various parameters of tumor-host relationship are being studied, like red blood cell dynamics and the mechanisms involved in the neoplastic anemias, the calcium metabolism of the host, and steroid hormone excretion patterns. This is done in an attempt to fathom the mode of action of the therapeutic agents and to seek the differences which exist between patients who do or do not respond to these agents.

Thus the scientists in our laboratories work on this tantalizing problem in a fairly successful effort to understand the chemical reactions of body cells. It is obvious that there must be some subtle change in the tumor cell which makes it divide so often, while normal cell division is so restrained. That difference has not been delineated, but it appears to us that our approach is a reasonable one which eventually may well give us some answer. It is also disconcerting that the therapeutic agents should so far have beneficial effects on particular cell types and have no influence on tumors of other origins. This too may be because we have not found the necessary formula.

But to have lived in this scientific era has been a great privilege. Now the chemical study of cells is being mastered and certainly we are finally at close grips with the problem, both in the laboratory and in the clinic. With all the public interest and support so generous at present we can only ask that we be given ample time, for this is a difficult and subtle abnormality which probably cannot be quickly unraveled. But the problem is worth the search and the tools needed for its study are at hand. Indeed, to solve it is certainly one of the world's major scientific goals. It is asking too much to expect this problem of cell division, so close to life itself, to offer its secrets for easy solution. Both scientists and the public need patience.

What I have said tonight refers to activities in our own laboratories—analogous reports could be made from other institutions in this country, as indeed from your excellent institution here.

Your handsome buildings and the work you are doing here are inspiring and I am grateful for this opportunity to see them.

STUDIES ON THE ENHANCEMENT OF THE MUTATION RATE BY CARCINOGENS

EDGAR ALTENBURG,
Rice Institute

Earlier work of Griffin had shown that 8-methoxy psoralen (a coumarin dye derivative) in combination with ultraviolet light was caricinogenic, though not by itself. It was therefore considered of interest to determine whether the two agents exert a parallel mutagenic effect. Present experiments indicate that psoralen alone, when applied to the polar cap cells (the germ cells in the developing egg) of Drosophila is non-mutagenic (2 lethal mutations in 496 chromosomes tested by Muller's "sifter" technique, which figure is about the control rate), but psoralen in combination with a very weak dose of ultraviolet (not sufficient by itself to be appreciably mutagenic) gave 19 mutations in 702 chromosomes, a rate (about $2.7 \pm 0.7\%$) well above the control rate. Urethane (a carcinogenic agent which interferes with mitosis and is mutagenic in Drosophila when fed to the developing flies and in bacteria but not in Neurospora) was found to be somewhat mutagenic when applied to the polar cap cells of Drosophila (22 lethals in 868 chromosomes, or $2.6 \pm 0.9\%$). Since feeding experiments involve many possible changes in the agent before it reaches the gonads of Drosophila, it was considered to be of interest to determine whether the agent was mutagenic when applied directly to the germ cells, as is done in the case of the polar cap method of treatment. The results of the present experiments are in conformity with the view that there is some connection between mutagenesis and carcinogenesis.

METABOLISM IN GERMFREE ANIMALS

THOMAS D. LUCKEY*

A germfree animal may be defined as an animal living in the complete absence of all demonstrable living microorganism. One can be sure he has a germfree animal only to the extent that his test shows no living microorganism present in or on the animal.

The idea of rearing germfree animals came directly from experiments in rearing legumes aseptically, in Pasteur's (1885) comments about rearing of peas and beans under aseptic conditions by Ducleau (1885). His statement of the problem is summarized: If I were a young man, I would try to rear animals germfree with the preconceived idea that life without bacteria would be impossible. After Nuttal and Thierfelder (1875), 1897) failed to rear either chicks or guinea pigs germfree, and Schottelius (1899, 1908) could get no growth in germfree chicks, Cohendy (1912) first successfully obtained growth in germfree chicks comparable to that of control chicks fed the same diet. This early work with germfree chicks provided a vehicle for developing apparatus, methods of sterilization, handling and testing of animals; and its success was the impetus to try to rear other species in the germfree state.

Rearing germfree chickens for three to six weeks in a simplified system can be done easily; it is one approach tried by several investigators. The equipment Pete Moore and I used at the University of Wisconsin in 1945–46 is shown in figure 1. The small jar is one of the simplest of germfree systems which adequately fulfills the requirements of a complete mechanical barrier isolating the sterile environment. Goats were obtained germfree and reared for a month in a sterile box by Kuster (1912) with no apparent ill effects.. Kuster appears to be the first to use a steam sterilization "clave" to bring objects to the germfree animals during the experiment. Reyniers and Trexler (1943) who have pioneered germfree techniques in this country, report other species

* From the Department of Biochemistry, School of Medicine, University of Missouri, Columbia, Missouri. Much of the data reported herein was taken from reports published or *in Press* from studies aided by the Office of Naval Research, Department of the Navy.

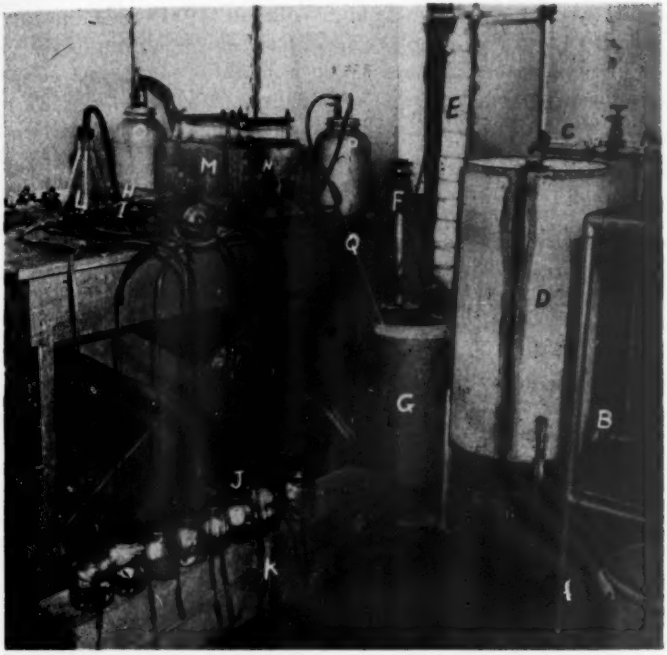


FIG. 1. Apparatus for Rearing Germfree Chicks (University of Wisconsin, 1945). The metal lined room has an antichamber with several large U.V. lights in the room, and in it. The room is steamed for one hour prior to aseptic transfers. (A) Prefiltered air under pressure enters (B) or bypasses (C), the humidifier (D) and goes into the heater (E), which is regulated to proper temperature (F) as it leaves the mixer (G). The air is sterilized by the glass wool-cotton filters (H) and goes through the manifold (I) to individual hatching units (J) and out through each tester (K). After hatch, sterile nutrient media (L) is sent through the manifold: it washes each chick and incubates at room temperature for 2 days in the bottom of the hatching unit. The tester is then pushed into the media and a sample is forced out where it is collected aseptically before contact with the outside air at point K. Microscopic examination of the media determines which chicks are sterile. The sterile chicks are aseptically transferred into the steam sterilized rearing unit (M) which is equipped with sterile air (N), food (O), water (P) and air outlet (Q) which depends upon air flow and dryness to maintain sterility.

which have been obtained in the germfree state are monkeys, rabbits, mice, and rats. The monkeys particularly grew well and were apparently easy to rear. Glimstedt has worked with germfree dogs but no details have been published. Guinea pigs appear to be hard to rear in the germfree state: Nuttal and Thierfelder

failed to rear them. Cohendy and Wollman (1914) and Glimstedt (1936) kept them alive for two months (some doubled their body weight in one month) and could apparently study the underdeveloped lymphatic system satisfactorily. Phillips, *et al.* (1955) used germfree guinea pigs in the study of amebiasis but gave little to indicate the health or growth of the animals.

Early attempts to raise germfree guinea pigs stimulated the development of apparatus with manipulation of feeding devices or animals as a major item. The bell-jar was first modified (Nuttal and Thierfelder, 1897), then abandoned for large boxes with gloves, viewing port, filters and a sterile entry "tunnel" (Glimstedt, 1936), and finally the boxes were made smaller to go into an autoclave by Gustafsson (1948), or made more sturdy for steam pressure sterilization by Reyniers (1943) and Glimstedt (1936). Figure 2 shows the Reyniers type unit. Reyneirs (1950) also developed a large sterile tank for which the operator dons a suit and enters the sterile environment through a germicidal bath. The aseptic operation of Kuster reminds one of the masterful aseptic techniques of Schottelius who used cubicles inside a series of sterilized rooms. These same principles are being used to set up germfree work in a sterilized room at the University of Missouri (Fig. 3). The basic design and principle of the self-sterilized room, which was established at the University of Wisconsin ten years ago, provides multiple physical barriers as a safety factor—the germfree cages are maintained in a sterile room.

This brief survey illustrates the germfree methods used, the animals available to past and present investigators, and provides a background for the work done, and that which might have been done. Actually few of the investigators were interested in biochemistry. Specific experimental studies have been made on the decreased lymphatic system in germfree pigs (Glimstedt, 1936), susceptibility of germfree guinea pigs to *Vibrio comma* (Cohendy and Wollman, 1922), the non-susceptibility of germfree guinea pigs to *Entamoeba histolytica* (Phillips, *et al.*, 1955), the effect of *E. coli* upon vitamin deficient germfree chicks (Balzam, 1938), and the production of dental caries in germfree rats by adding known cultures of bacteria (Orland, *et al.*, 1955). When attempting to rear each species, most investigators necessarily had to study the nutrition of the animals with which they have

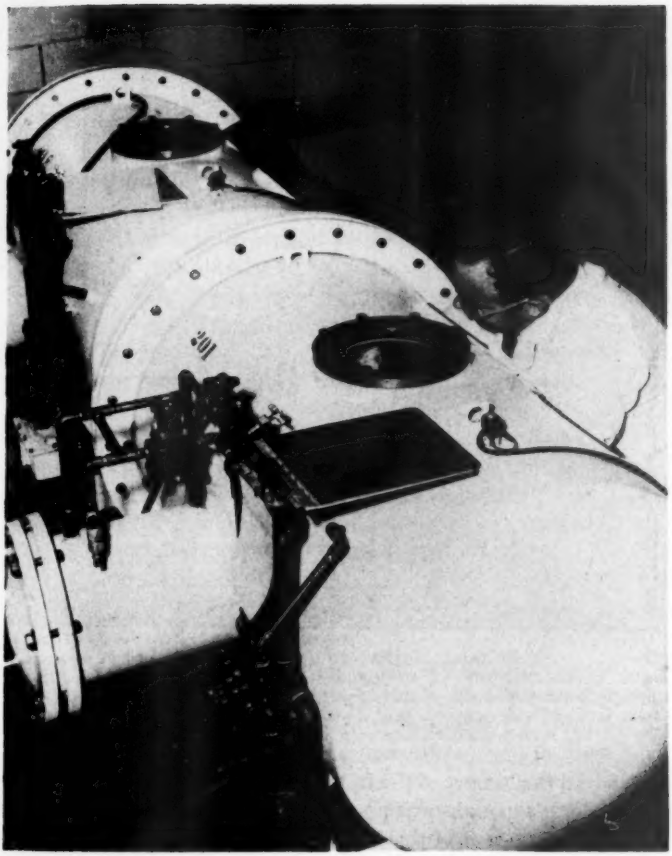


FIG. 2. Reyniers type germfree tank, series 200. This unit has a viewing glass and arm-length gloves gasketed into it prior to steam sterilization. The pipes contain the air inlet filters and the air outlet system. The "tunnel" on the side has doors inside and outside which are both closed when food, water or utensils are steam sterilized before being taken into the germfree environment. A special attachment is used to take eggs through a germicidal trap into the unit. An operating cage may also be connected to obtain germfree rats via cessarian section. These are handfed until weaning in much the same manner as shown by the picture of a swab being inserted into the rat's mouth. Reyniers *et al.* (1949) have described the detailed use of this apparatus.

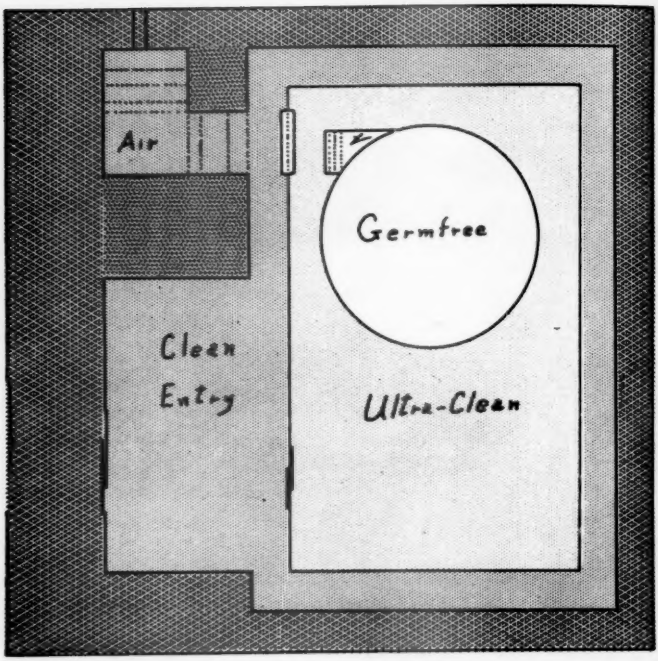


FIG. 3. Sterile Room at the University of Missouri. The germfree cage is located in an ultra-clean, self-sterilizing room. The self-sterilization is attained through the case of banks of ultra-violet lamps, positive (sterile) air pressure, steam jets and a germicidal floor. This allows a better opportunity to maintain sterility within the germfree unit.

worked. In fact, many of the failures in rearing germfree animals have been due to malnutrition.

This presentation of the metabolism studies which have been reported in the literature gives insight into the character of the germfree animal and illustrates its usefulness as a tool for the direct study of infectious diseases, intestinal synthesis, mechanisms of defense, and purely metabolic diseases (of which cancer may be an example) which lie beyond the germ theory. Germfree animals may be used to separate metabolic disease, or the metabolic aspect of a disease, from infectious disease. An example of this is the absence of dental caries in germfree rats until one or more cultures of bacteria were added. Surprisingly, a culture of

lactic acid bacteria produced few, small carious lesions (Reyniers, 1953) while species with high proteolytic activity produced large lesions consistently (Orland, *et al.*, 1955). Evidently homofermentive lactics might retard caries by displacing oral proteolytic bacteria.

Gross Observation

Guinea Pigs. Nuttal and Thierfelder (1897) reared germfree guinea pigs which had inflated ceca and gained only 23 g. in 13 days. The ceca were distended and filled with a brown liquid. They thought the absence of bacteria was good for the animal. Glimstedt (1932) kept guinea pigs germfree for 2 months. They grew as well as controls fed the same diets. He reported the lymphatic system was grossly underdeveloped. Incidentally, he suggested the metabolism of germfree animals as a good problem. He found the whole lymphatic system was atrophied, the parenchyma was rarefied and the lymphocytes were reduced in the lymph nodes with practically none in the mucous membrane of the intestine. The guinea pigs reported by Reyniers (1946) were obviously starving and died at about 60 days. He reports an absence of rigor mortis in these animals. (Rigor mortis has been seen in germfree chicks by the author.)

Rats. Gustafsson (1946) reported that germfree rats had reduced thymus and lymphatic organs (½–⅓ that of conventional rats) and mentions that for 30 days hand-fed germfree rats do not grow as fast as their control dam-fed rats. In 1948 he reported growth comparable to other hand-fed rats; his rats also showed distended ceca and small lymph glands. Blood and prothrombin clotting times were very long.

Reyniers (1953) reported reproduction in germfree rats through 7 generations. Details of this important colony have not yet been published. Reyniers, *et al.* (1946), suggest that the suckling germfree rats need vitamin C in the diet for survival. Data presented from older germfree rats is disregarded here because, as given in the text, these rats were contaminated prior to examination. This was reflected in the high white blood cell counts. Gordon (1955) reported germfree animals were generally the same as conventional animals in terms of morphology, growth, red cell count, reproduction and longevity; in general health was normal. He found the weight of the gastrointestinal tract to be less per 100

gm. germfree rat when compared to normal rats; this is due to decreased connective tissue and lymph apparatus in the wall. He emphasizes that the low white cell count in the blood is a reflection of the decreased production in the lymph nodes (particularly are the intestinal nodes much decreased) combined with normal production by the spleen and thymus.

The growth rate of male rats in the germfree state is less than expected, while that of germfree females appears to be similar to that of contaminated female rats (Fig. 4). The germfree rat appeared to require no added vitamin K when fed a synthetic type diet. It is entirely possible that traces of this vitamin in the diet would satisfy the requirements for vitamin K. This finding is in direct contradiction to the findings of Gustafsson. However, Luckey, *et al.*, (1954) found vitamin E prevents hemorrhages when germfree rats are fed a low protein diet. The germfree rat appears to have a dietary requirement for biotin and not folic acid.

Chicks. Reports of the past few years (Luckey, *et al.*, 1948; Reyniers, *et al.*, 1950; Reyniers, *et al.*, 1949) indicate that germfree chicks grow, feather and develop "normally' when compared

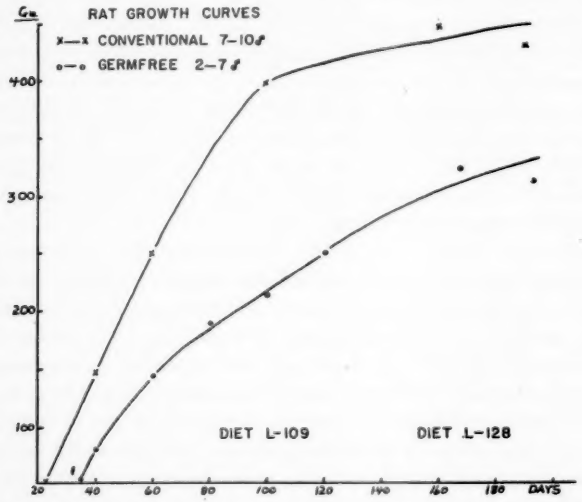


FIG. 4. Composite Rat Growth Curves (Luckey, *et al.*, 1955 and Orland, *et al.*, 1954).

TABLE 1

*White Leghorn Chicks**

Tissue		Dry wgt.	% Ash, dry	% N_2	% Fat	Flavin	Niacin	Ca pan.	Biotin	Folacin	B_{12}
Whole	G.F.	*29.6*	11.8	...	...	4.25	*45.2*	26.0	0.070	6.95	...
Chick	C.	*33.7*	10.0	...	...	4.62	*54.6*	22.2	0.064	6.02	...
	G.F.	*26.2*	*6.01*	3.09	4.58	21.5	108	62.5	5.24	4.49	323
Liver	C.	*29.2*	*4.66*	2.83	5.77	23.3	109	48.0	4.74	3.37	*152*
	G.F.	...	...	...	...	...	*2.62*	0.893	0.0467	0.720	2.86
Blood	C.	...	...	...	...	...	*0.0*	1.17	0.0607	0.723	4.21
	G.F.	22.2	6.39	2.96	2.45	8.11	33.5	17.5	3.00	0.86	15.1
Muscle	C.	22.9	5.39	2.98	2.04	10.9	46.5	14.3	2.83	1.06	15.6
	G.F.	...	66.6	2.07	21.2	14.3	32.0	5.93	1.69	5.97	5.99
Bone	C.	...	64.4	...	22.2	12.8	20.5	5.23	0.915	1.83	3.43
	G.F.	18.6	*10.7*	1.64	6.23	2.33	25.4	*55.3*	0.88	0.533	15.1
Brain	C.	19.9	*7.55*	1.73	6.41	2.10	21.8	*64.8*	1.11	0.550	16.3
	G.F.	*22.9*	11.6	0.716	...	*48.2*	7.6	3.05	*0.237*	0.36	6.00
Bile	C.	*24.9*	12.5	0.703	...	*12.7*	8.8	2.30	*0.07*	0.40	3.86
Cecal	G.F.	*13.0*	*20.3*	0.817	2.81	4.9	*13.6*	*34.3*	0.760	1.36	14.2
Contents	C.	*19.1*	*24.3*	1.15	3.33	8.9	*48.4*	*21.9*	1.59	2.24	18.4
Diet		90	6	...	6.2	9.3	83.1	30.4	0.35	2.5	29.9

* This data is detailed in Lob. Rept. No. 3, in press.
Three or more chicks per group; the liver and cecal contents data came from 8-31 chicks each. The chicks were 32-360 days old.
The vitamins are expressed as γ/gm, wet basis, except vitamin B_{12} is mγ/gm.
GF = germfree chicks. C = conventional chicks. Italics indicates $p = <0.03$.

to conventional clutch-mate chicks fed the same steam sterilized diet. Reproduction has also been attained. This supports the conclusions of Cohendy and Balzam. Perosis is occassionally seen in germfree White Leghorn chicks, and rarely in White Wyandotte Bantam chicks. This indicates not only genetic differences but shows definitely that perosis is a metabolic disease which involves little or no microbial participation. Blood studies of germfree chicks indicate good hemoglobin and red cell formation with a lowered white cell count. These germfree chicks had smaller livers (a phenomena seen when chicks are fed synthetic-type diets but not when fed practical diets), lymph nodes, ceca and intestinal tracts than ordinary chicks. The spleens and thymuses were very well developed. Thymocyte production was slightly above that of ordinary chicks (Gordon, 1955). Certainly these chickens must then metabolize primarily in the same pathways as ordinary birds.

Analysis of chicken organs (Table 1) shows very few major differences. Many of the differences marked as statistically significant have no practical meaning to the biochemist, *e.g.* 26γ vs. 22γ pantothenate per gm. of germfree and conventional chickens respectively. The germfree chick tends to carry more moisture in its tissues. The bile of the germfree chicks contains considerably more riboflavin and biotin than is found in ordinary chicks. Also germfree chicks appear to have higher concentrations of vitamin B_{12} in the liver, and niacin in the blood than their multi-contaminated clutch-mates.

Microorganisms evidently do not change the pH of the gastrointestinal tract (Table 2) (Reyniers, *et al.*, 1956).

About as close as I can come to cancer in this report to you is to mention that occasional hatches of germfree chicks get a disease

TABLE 2

pH in White Leghorn Chicks

	G.F. (3)		Conventional (2)	
Mouth	7.54	(7.0–8.5)	6.54	(6.5–6.6)
Crop	6.15	(5.9–6.3)	6.35	(6.3–6.4)
Proventriculus	4.81	(4.6–4.9)	5.63	(5.6–5.7)
Gizzard	4.30	(3.3–5.0)	3.47	(3.4–3.5)
Duodenum	6.42	(6.3–6.6)	6.38	(6.3–6.5)
Cecum	6.38	(6.3–6.5)	6.48	(6.3–6.7)
Colon	6.26	(6.1–6.5)	6.37	(6.2–6.5)

appropriately designated as "jitters" (Reyniers, 1949 and 1953). Conventional (or contaminated) chicks hatched from the same clutch of eggs have never shown this syndrome, whereas if one germfree chick "got the jitters" all those clutch-mates in the same cage "caught" the disease either at the same time or within one week from the time symptoms were seen in the first chick. The symptoms noted were (1) general weakness, (2) slight body tremors and an unsteady gait, (3) coarse tremors ("jitters") and a jittery gait, and (4) death. Vitamin deficiencies were first suspected since thiamin, pyridoxine or vitamin E deficiencies may show similar characteristics. Vitamin therapy failed completely. Upon post mortem examination Dr. Gordon found a meningeal proliferation in the region of the cerebellum in some of the chicks. Evidently some change in metabolism, the presence or absence of some metabolite in abnormal quantity, allows this virus, or virus-like, manifestation to exert itself in the absence of (other) microorganisms.

Turkeys. A limited number of Beltsville White Turkey poults were reared in experiments which indicated that germfree turkeys grow somewhat faster when fed antibiotics than when fed no antibiotic (Luckey, *et al.*, 1956). A comparison of the conventionally reared turkeys to germfree turkeys (Luckey, *et al.*, in press) indicate they are practically identical in gross appearannce and growth with the possible exception that the snood of the germfree male may be somewhat larger than that of the conventional male poult. The growth comparison of male turkey poults fed a steam sterilized synthetic-type diet is given in figure 5.

Metabolism Studies

Rat. Rat metabolism studies indicate the gross relationship of the intestinal microorganisms to the host. Germfree and contaminated rats eat and drink about the same quantity per 100 gm. body weight. Yet the feces of germfree rats contain essentially no microorganism while the conventional rat fecal material has been estimated to contain 50–90 per cent microorganism and their by-products. This gives a suggestion that the micro flora may not affect the net intake and output of food and feces. Microbes live in the gastrointestinal tract simply because it presents a well regulated incubator with lots of food in the fluid. It is possible

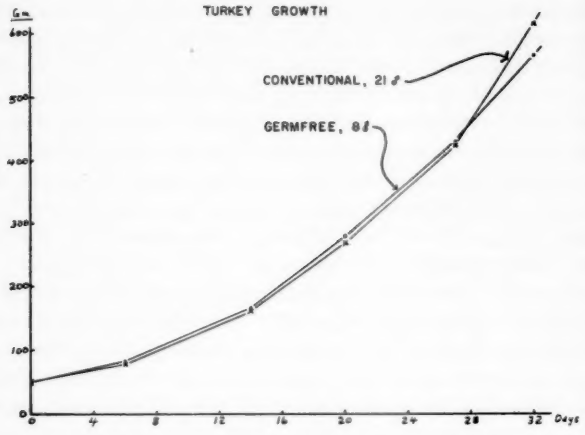


FIG. 5. Turkey Growth Curves (Luckey, *et al.*, 1956).

that the net result of "intestinal synthesis" may be disregarded as a major nutritional factor.

A complete vitamin balance study was run in rats fed a synthetic type diet for five months (Luckey, *et al.*, 1955). The results, figure 6, indicate 3 general states of vitamin metabolism. In one, illustrated by niacin, riboflavin and biotin, less of the vitamin is found in the excreta, wasted food, lost hair and carcass than was put into the system via food and initial tissue stores. These vitamins are evidently destroyed or changed during metabolism to forms (*e.g.* trigonellin from niacin) which are inactive in the microbiological assay. In the second state quantities of the vitamin were found which were about equal to the amount put into the rat-diet system. Pantothenic acid and vitamin B_{12} appear to be in this category. In both of these metabolic states only the overall balance is seen; a vitamin could conceivably be synthesized in large amounts by the tissues and the destruction of that vitamin could be great enough to indicate no net synthesis in this system. Radioactive precursors would be needed to show the existence of biosynthesis of such a vitamin. In the third state of vitamin metabolism, more of the vitamin was found in the excreta-waste carcass system than was put in via the food-tissue system. Vitamin B_9 (as measured by folic acid and citrovorum

factor) appears to typify this condition. The data presented in figure 7 show that little folic acid was excreted by biotin-deficient, germfree rats. When biotin was administered, the folic acid excretion increased tremendously. One might thus postulate that biotin is a cofactor in folic acid biosynthesis. These two vitamins are often involved together in various syndromes, *e.g.* the sulfa-fed rat; and now we see something of the reason why there is a very direct dependence of the folic acid metabolism upon the biotin present.

Gordon (1955) reports that the adrenal glands of germfree and conventional rats have similar quantities of ascorbic acid and cholesterol. These tests of function corroborate studies in morphology to show the adrenal gland in the germfree animal is "normal."

In an attempt to determine whether germfree rats could synthesize biologically labile methyl groups, du Vigneaud, *et al.* (1951) fed 10 per cent deuterium water to rats for a few days, then continued with 3 per cent deuterium water with a diet complete except for the omission of choline. A summary of their re-

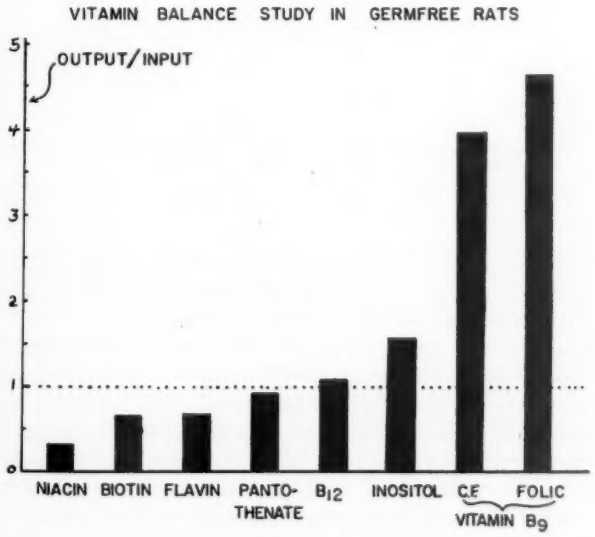


FIG. 6. (Luckey, *et al.*, 1955b).

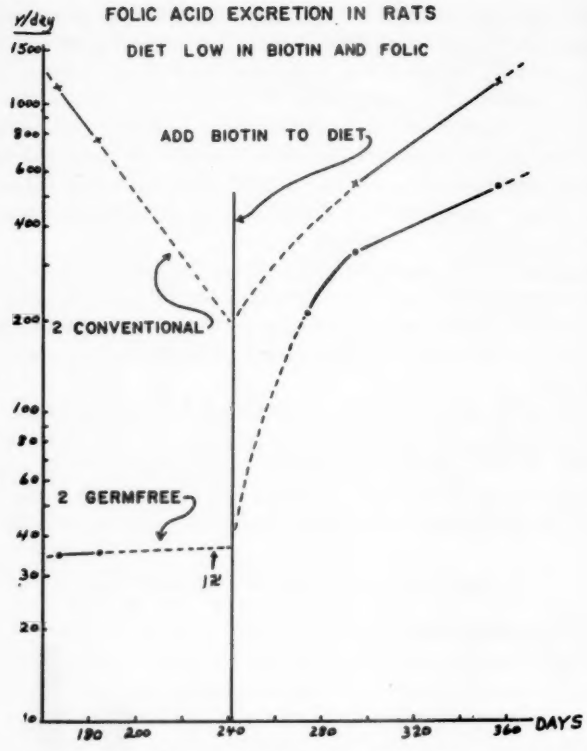


FIG. 7. (Luckey, *et al.*, 1955b).

sults (Table 3) shows both germfree and conventional rats had the same amount of choline in their tissues and in both groups the choline carried deuterium in the methyl group.

Goats. Kuster (1912) found NH_3 of urine decreased in germfree goats which was counteracted by a rise in urea excretion.

Chicks. Germfree work has highlighted the existence of a new biological phenomenon which may be called "bio-incrassation." Much of the evidence cited to show the intestinal synthesis of vitamins can be attributed to bio-incrassation. Table 1 shows how the vitamin concentration is increased 5–10 fold (dry basis) as it passes through the intestine. Part of this increased concentration

is evidently caused by the adsorption of relatively larger quantities of fat, carbohydrate and protein than of some vitamins (or minerals). When this phenomenon was seen in conventional animals it was interpreted as "intestinal synthesis" of vitamins (by microorganisms in the tract); but its occurrence in germfree animals negates that as the sole interpretation and leaves only the possibilities of excretion from the tissues into the lumen and/or

TABLE 3

Methyl Synthesis from D_2O (10 4 Percent) Adult Rats

	Rat No.	Wgt. gm.	Mg. Choline per 100 gm. body wgt.	Deuterium Content	
				Body water atom percent excess	Me group of Choline atom excess
Conventional	1	253	0.048	2.20	0.21
	2	282	0.047	2.27	0.20
Germfree	5	322	0.047	2.44	0.16
	6	289	0.048	2.99	0.10

TABLE 4

M. Smegmatis—M. Wagner

GP	Material	Aver. No./100 Fields
1	Diet	0
1	Feces	0
2	Diet + Bacteria	131
2	Feces	870
2	Feces (36 hr.)	3

bio-incrassation. The effect is shown more clearly in the data of Wawner (see Reyniers, *et al.*, 1949) reproduced in Table 4 using an easily identified bacterium fed to germfree chicks. Feeding this organism at level of 130 per 100 fields resulted in fecal concentration of 870 per 100 fields.

Wagner (1955), who has studied the blood proteins of germfree chicks via serological reactions, reports germfree chicks have a natural heterohemagglutinin for rabbit erythrocytes. Germfree chicks have no natural antibacterial agglutinin when tested with *Paracolobactrum aerogenoides*, *Escherichia coli* or *Micrococcus epidermidis* during their first half year of life. About this time small reactions may occur from transient dead bacteria present in

the diet. Agglutination was not obtained from organisms not found in the diet. Large quantities of dead bacteria were found to produce measurable serum titers much faster. When dead *Salmonella pullorum* or beef serum were injected into germfree chicks the titers were almost identical with those obtained by injecting conventional chicks to show that the antibody producing aparatus of the body lacked only a specific stimulus.

The vitamin A, thiamin, riboflavin, pantothenate acid, folic acid content of the livers of four week old germfree chicks was equal to that of conventional chicks while the ascorbic acid content was somewhat low (Reyniers, *et al.*, 1950). The adrenal ascorbic acid appeared to be the same in both groups. The B-vitamins in the cecal contents of germfree and conventional chicks were usually in the same order of magnitude. Variations on this theme are seen in both tables 1 and 6. Some of the vitamins showed a decreased concentration in the liver as the chick aged. These effects are noted in figure 8.

Balzam (1937) showed germfree chicks suffered from vitamin "B" deficiency in much the same manner that conventional chicks do. This work was lately confirmed (Luckey, *et al.*, 1955) when germfree chicks were obtained deficient in nicotinic acid, folic acid, riboflavin and thiamin (each as a separate series of experiments). In all of these (Fig. 9) except thiamin deficiency, the gerfree chicks began to die before the conventional chicks. This, combined with the growth curves, give indirect evidence of sources of the viatmin available to the conventional animal but not available to the germfree chick. Possible sources include dust in the air, more vitamin in water or food via microorganism synthesis as well as intestinal synthesis.

One of the most interesting aspects of vitamin metabolism occurred in the thiamin deficient birds. When the chicks were moribund or freshly dead from a thiamin deficiency, analyses were

TABLE 5

Thiamin in Bantam Chicks at 11 + 3 Days Aver. Values γ/gm (Dry Basis)

	Diet	N	Liver	N	Cecal	N	Rectal
Germfree	4	2	21.6	2	52.7	.	...
	< 0.01	3	4.6	7	34.0	1	106
Conventional	4	4	15.8	4	109	1	200
	< 0.01	4	8.5	2	43.6	3	183

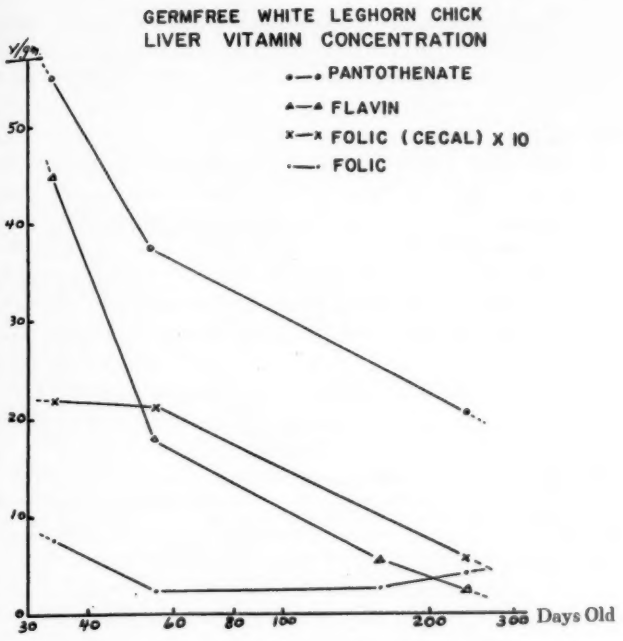


FIG. 8. (Reyniers, *et al.*, 1956).

made as shown in the table 5. Both germfree and conventional deficient chicks had lower concentrations of the liver than was found in chicks fed a complete diet (about 400γ of thiamin per 100 gm. diet) and less in their excreta. However, even while the germfree chicks were dying of thiamin deficiency they were excreting a large amount of thiamin. The cecal contents had 7 times more thiamin than the liver of the deficient germfree chicks. Similar, if less dramatic, circumstances existed for riboflavin, niacin, and folic acid deficiencies. These data indicate the body could not effectively compensate for deficiency of a nutrient such as thiamin.

Another interesting facet found in vitamin metabolism was the apparent spontaneous recovery of germfree chicks from a vitamin K deficiency (Fig. 10). It would appear that vitamin K is superceded by another metabolite, or else the body has learned to syn-

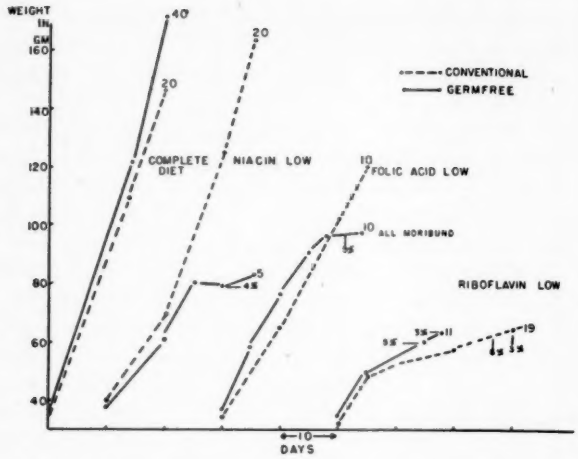


FIG. 9. Vitamin deficiencies in chicks. (Luckey, *et al.*, 1955a).
Growth curves of White Leghorn chicks fed semi-synthetic diets. The number of chicks which finished the experiment is given at the end of the curves indicating the average growth obtained for 2 to 5 experiments. The numbers preceding the swastikas indicate the death of deficient chicks.
Permission is granted by Wistar to use this reproduction.

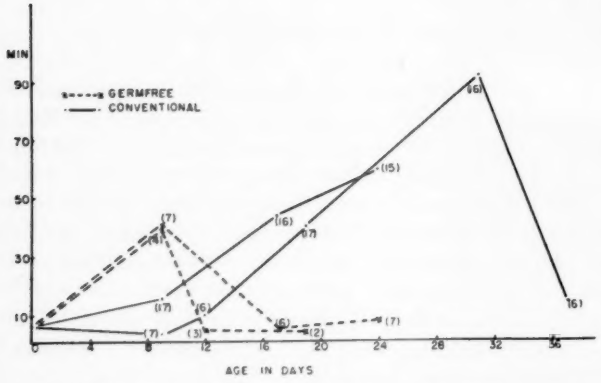


FIG. 10. Vitamin K deficiency (Luckey, *et al.*, 1955a).
Whole blood clotting time for White Leghorn chicks fed the diet of Ansbacker in two experiments. The numbers of chicks used to obtain each average are indicated in parentheses. Some of the 10 chicks examined to obtain data at hatch were taken from the shell after pipping.
Permission is granted by Wistar to reproduce this figure.

thesize either a vitamin K active compound or a compound which was previously made only in the presence of vitamin K.

Vitamin metabolism was further studied in germfree chicks by analyzing liver and cecal contents for individual B-vitamins when chicks were deficient in a number of different vitamins. The data summarized in table 6, show that the concentration of most vitamins is relatively stable. Thiamin concentration was never significantly different when germfree and conventional birds were compared. When fed a thiamin deficient diet or a folic acid deficient diet, the liver thiamin decreased ($p = <0.001$ in each) in both germfree and conventional chicks, except the decrease seen in conventional chicks fed a folic-low diet was not significant ($p = 0.27$).

No differences in riboflavin concentration were noted on the thiamin deficient diet. When the chicks were fed a riboflavin deficient diet, all the values reported for the riboflavin content of tissues were lower than those of tissues from birds fed a complete diet. When a niacin deficient diet was fed, the fiavin concentration in germfree chicks liver tended to increase ($P = 0.12$) over that of conventional chicks and it was definitely higher ($p = <0.001$) than the concentration of flavin in livers of germfree chicks fed a complete diet. At the same time the flavin content of the germfree cecal contents did not decrease below that found in chicks fed the complete diet ($p = 0.18$). It was, however, lower than the riboflavin content of the conventional chicks ($p = 0.004$). The riboflavin in the liver of germfree chicks fed a folic acid low ration was high when compared to conventional chicks fed the same diet ($p = 0.002$) or to germfree chicks fed a complete diet ($p = <0.001$). The riboflavin concentration of liver and cecal content of both germfree and conventional chicks decreased ($p = <0.001$ to 0.011) when a diet low in vitamin K was fed. The presence of menadione in the diet evidently allows either less adsorption of riboflavin from the intestine (relative to the major nutrients), greater excretion of riboflavin into the lumen (via the bile and/or the succus intericus), or both. In the germfree chicks niacin and folic acid seems to have the opposite effect from vitamin K on decreasing the flavin concentration of the cecal contents. It would be interesting to learn how added menadione or decreased dietary niacin or folic acid affects increased storage or riboflavin in the liver.

TABLE 6

B Vitamin Metabolism in Chicks
(39–61 days old)

Diet	Thiamin		Flavin		Niacin		Calpan		Biotin		Folic	
	GF	C	GF	C	GF	C	GF	C	GF	C	GF	C
CECAL CONTENTS												
Complete	...	...	10.9	7.38	12.1	37.2	5.47	7.74	0.241	0.488	2.29	1.82
Flavin low	1.70	1.94	..	3.3	...	...	...	8.0	2.4	0.15	...	1.1
Niacin low	0.464	...	5.0	9.4	14.6	37.2	15.2	11.7	0.21	0.38	2.1	0.74
Folic low	...	...	5.4	4.6	7.8	8.1	...	2.4	...	...	0.55	1.22
Vitamin K low.	...	...	3.6	2.6	25.0	19.1	16.2	3.88	0.35	0.49	1.05	0.67
LIVER CONTENTS												
Complete	3.99	3.00	21.6	23.3	109	108	66.4	48	5.14	4.31	4.41	3.27
B_1 deficient	0.824	0.647	17.7	25.0	69.3	104	47.6	45.4	4.47	4.70	3.7	2.96
Flavin low	3.27	3.86	7.1	12.5	86	123	57.7	47.3	4.3	4.0	5.2	2.8
Niacin low	4.94	4.26	34.7	22.7	145	110	54	51	5.1	6.5	2.9	2.3
Folic low	2.49	2.12	34.9	19.2	104	87	19.3	13.7	4.15	3.49	3.60	2.31
Vitamin K low.	4.52	4.84	16.1	10.9	130	141	19.6	17.9	4.9	3.7	5.0	3.4

GF = germfree chicks. C = conventional chicks.
Analyses are expressed as γ/gm on the wet weight basis.

A study of niacin showed considerably more niacin in the cecal contents of conventional chicks than in germfree chicks ($p = 0.003$) when both were fed a complete diet. The only other statistically significant difference found in niacin concentration of the cecal contents was the decrease seen in conventional chicks when a folic acid-low diet was fed ($p = 0.022$), although the decrease in the absence of menadione approached significance ($p = 0.041$) when compared to values from conventional chicks fed a complete diet. When a riboflavin-low diet was fed, the concentration of niacin in the liver of germfree chicks decreased significantly below the level found in either conventional chicks fed the same diet ($p = 0.016$) or in germfree birds fed a complete diet ($p = <0.001$). Surprisingly, when a niacin-low diet was fed no decrease in niacin content was noted—the niacin concentration in germfree chick liver increased significantly above that of conventional chicks fed the same diet ($p = <0.001$) and above that of germfree chicks fed a complete diet ($p = <0.001$). This phenomenon occurred while the germfree chick became moribund and died: the conventional chicks showed little evidence of any deficiency (see growth curves during niacin deficiency in figure 8). The liver concentration of niacin increased when viatmin K was omitted from the diet of germfree birds ($p = 0.023$) but variation kept the difference from being significant in conventional chicks. When a folic acid deficient diet was fed, the concentration of niacin in the cecal contents decreased significantly in conventional chicks ($p = 0.022$) but not in germfree chicks.

When fed a vitamin K deficient diet, germfree chicks showed increased pantothenic acid in the cecal contents when compared to germfree chicks fed a complete diet ($p = <0.001$) or conventional chicks fed the same diet ($p = 0.016$). Conventional chicks fed the vitamin K deficient diet tended to maintain less pantothenic acid in their cecal contents than did conventional chicks fed a complete diet ($p = 0.046$). The pantothenic acid concentration of the liver in chicks fed a complete diet was higher in germfree chicks than in conventional chicks ($p = 0.001$). The liver concentration of pantothenate was lowered in germfree and conventional chicks fed a diet deficient in either folic acid or vitamin K ($p = <0.001$ in all 4) when compared to chicks receiving the complete diet.

The biotin concentration in tissues appears to be more constant than other vitamins. The only significant change was the increase in biotin concentration of cecal contents when a riboflavin deficient diet was fed to germfree chicks as compared to that of conventional chicks fed the same diet ($p = <0.001$), or when compared to germfree chicks fed a complete diet ($p = <0.001$).

Many small changes in folic acid metabolism noted were statistically significant because there was extremely small variation between values obtained under each condition. Thus, the concentration of folic acid in the liver of germfree chicks proved to be higher than that of conventional chicks when both groups were fed a complete diet ($p = 0\ 021$), a folic acid deficient diet ($p = 0.022$), and possibly a niacin deficient diet ($p = 0.044$). In conventional chicks the folic acid concentration of the liver was higher in chicks fed a complete diet when compared to those of chicks fed a folic acid deficient diet ($p = 0.016$), or a niacin deficient diet ($p = 0.032$). In the latter situation the folic acid concentration of germfree chicks liver had a tendency to be lower than that of germfree chicks fed a complete diet ($p = 0.079$). In all cases, except one, the folic acid concentration of cecal contents was lower when a deficient diet was fed than when the complete diet was fed ($p = 0.02 — 0.001$). The exception was that the concentration of folic acid in cecal contents of germfree chicks fed a niacin diet did not decrease significantly. Omitting explanations which involve the intestinal microfloral (since they cannot be the only cause), one is led to suggest that this effect may be due to a general decrease in food efficiency in the deficient birds. In other words intestinal bio-incrassation of folic acid is less active in sick animals than it is in normal animals. With several exceptions, this is a general pattern for other vitamins. Unfortunately, data have not been obtained to examine this possibility.

Summary

In summary, the germfree technique has developed over a period of sixty years using a variety of animals. The apparatus may be as simple as a canning jar or a churn jar; it may be a large autoclave-type unit; or it may be a whole room: in each case a mechanical barrier separates the sterile environment from the non-sterile environment. The experimental vertebrates which

are available for investigators today are: chicks and other birds such as the turkey, rats, guinea pigs, dogs, rabbits, monkeys, and goats. While all investigators have had to solve problems in feeding these animals, few have studied metabolism in germfree animals.

Morphologically, germfree animals closely resemble contaminated animals; differences are that the germfree animals have under developed lymphatic systems, often an enlarged cecum in mammals, a slightly smaller gastrointestinal tract, and on some diets the liver is smaller. Chemically the germfree animals can probably not be separated from conventional animals in terms of gross analysis or composition. One consistent difference is the lack, or slow development, of acquired serum antibodies sensitive to specific bacterial antigens; certain natural antibodies do exist.

The nutritional requirements of germfree animals are qualitatively the same as those of conventional animals with the exception that the germfree rat requires biotin. Quantitative studies have not been made. Germfree chicks fed diets deficient in certain B-vitamins, particularly niacin, show more acute deficiency symptoms and die sooner than do conventional chicks fed the same diets. Studies in vitamin interrelationships indicate that biotin is one of the most stable of B-vitamins in the tissues while niacin, riboflavin and pantothenate show many variations.

The germfree rat can form methyl groups in its tissues and appears to require biotin for the bio-synthesis of vitamin B_9 (folic acid and citrovorum factor).

REFERENCES

Balzam, M.: Elevage aseptique des animaux. I. Appareillage et methode. II. Elevage aseptique des poules maintenue aux regimes complets et deficients. *Ann de Physiol*, 13: 370–385, 1937.

Balzam, M.: Aseptiche Zucht von Tieren. I. Apparatus and methods. II. Aseptiche Zucht von Hahuern mit Vitamin Haltzen and Vitamin freien Futter. *Acta. Biol. Esper* (Warsaw), 11: 43–56, 1938.

Cohendy, M.: Experiences sur la vie sans microbes. *Ann. Inst. Pasteur*, 26: 106–137, 1912.

Cohendy, M., and E. Wollman: Experiences sur la vie sans microbes. Elevage aseptique de cobayes. *C. R. Acad. Sci.*, 158: 1283, 1284, 1914.

Cohendy, M., and E. Wollman: Quelques resultats alquis par la methode des elevages aseptiques. I. Scorbut experimental. II. Infection cholerique du cabaye aseptique. *C. R. Acad. Sci.*, 174: 1082–89, 1922.

Ducleau, M. E.: Sur la germination dans une sol riche en mateires organiques, mais exempt de microbes. *C. R. Acad. Sci.*, 100: 66–68, 1885.

Glimstedt, G.: Das Leben ohne Bakterien. *Anat. Anz.*, 75: 79–89, 1932.

Glimstedt, G.: Bakterienfreie Meerschweinchen. *Acta. Pathol and Microbiol. Scand. Suppl. XXX*, 1–295, 1936.

Gordon, H. A.: Germfree Research. A Basic Study in Host-contaminant Relationship. III. Morphologic Characterization of Germfree Life. *Bull. N.Y. Acad. Med.*, 31: 239–242, 1955.

Gustafsson, B.: Magra Erfarenheter fran Bakteriefri uppfodning av rattor. *Nord. Med.*, 32: 2665, 1946.

Gustafsson, B.: Germ-Free Rearing of Rats. *Acta. Path. et Microb. Scan., Suppl. LXXII*, 1–130, 1948.

Kuster, E.: Die Keimfreie Zuchtung von Saugetieren und ihre Bedeutung fur die Erforschung der Korper funktionen. *Centrable f. Bakterial*, 54: 55–58, 1912.

Luckey, T. D., H. A. Gordon, M. Wagner, and J. A. Reyniers: Growth of Germfree Birds Fed Antibiotics. *Antibiotics and Chemotherapy*, 6: 36–40, 1956.

Luckey, T. D., J. R. Pleasants, and J. A. Reyniers: Germfree chicken Nutrition. II. Vitamin Interrelationships. *J. Nutr.*, 55: 105–118, 1955a.

Luckey, T. D., J. R. Pleasants, M. Wagner, H. A. Gordon, and J. A. Reyniers: Some Observations on Vitamin Metabolism in Germ-Free Rats. *J. Nutr.*, 57: 169–182, 1955b.

Luckey, T. D., J. A. Reyniers, P. Gyorgy, and M. Forbes: Germfree Animals and Liver Necrosis. *Ann. N.Y. Acad. Sci.*, 57: 932–935, 1954.

Luckey, T. D., M. Wagner, H. A. Gordon, and J. A. Reyniers: Rearing Germfree Turkeys. *Lobund rept.*, (in press).

Nuttal, G. H. F., and H. Thierfelder: Thierisches Leben Ohne Bakterien im Verdauungskanal. *Z. Physiol. Chem.*, 21: 109–121, 1896.

Nuttal, G. H. F., and H. Thierfelder: Thierisches Leben Ohne Bakterien im Verdauungskanal III. *Zs. Physiol. Chem.*, 23: 231–35, 1897.

Orland, F. J., R. J. Blayney, R. W. Harrison, J. A. Reyniers, P. C. Trexler, R. F. Ervin, H. A. Gordon, and M. Wagner: Experimental Caries in Germfree Rats Inoculated with Enterococci. *J. Am. Dental Assoc.*, 50: 259–272, 1955.

Orland, F. J., J. R. Blayney, R. W. Harrison, J. A. Reyniers, P. C. Trexler, M. Wagner, H. A. Gordon, and T. D. Luckey: Use of the Germfree Animal Technique in the Study of Exeprimental Dental Caries I. Basic Observations on Rats Reared Free of All Microorganisms. *J. Dental Res.*, 33: 147–174, 1954.

Pasteur, L.: Observations relative a la note de M. Duclaux. *C. R. Acad. Sci.*, 100: 68, 1885.

Phillips, B. P., P. A. Wolfe, C. W. Rees, H. A. Gordon, W. H. Wright, and J. A. Reyniers: Studies on the Ameba-Bacteria Relationship in Amebiasis Comparative Results of the Intracecal Inoculation of Germfree, Monocontaminated and Conventional Guinea Pigs with Entamoeba Histolytica. *Am. J. Trop. Med. Hyg.*, 4: 675–692, 1955.

Reyniers, J. A.: Introduction to the General Problems of Isolation and Elimination of Contamination. Micrurgical and Germ-Free Techniques. C. C. Thomas, Springfield, Illinois, 1943.

Reyniers, J. A.: Biological Apparatus. U.S. Patent No. 2, 516, 419, July, 1950.

Reyniers, J. A.: The Significance of Germfree Life Methodology (Gnotobiotics) to Experimental Biology and Medicine. *M.S.C. Veterinarian*, 13: 178–185, 1953.

Reyniers, J. A.: Germ-Free Life Applied to Nutrition Studies. *Lob. Rept.*, 1: 87–120, 1946.

Reyniers, J. A.: Some Observations on Rearing Laboratory Vertebrates Germfree. *Proc. N.Y. St. Assoc. Pub. Health Lab.*, 28: 60–69, 1949.

Reyniers, J. A., and P. C. Trexler: The Germ-Free Technique and Its Application to Rearing Animals Free from Contamination Micrurgical and Germ-Free Techniques. C. C. Thomas, Springfield, Illinois, 1943.

Reyniers, J. A., P. C. Trexler, and R. F. Ervin: Rearing Germ-Free Albino Rats. *Lob. Rept.*, 1: 1–84, 1946.

Reyniers, J. A., P. C. Trexler, R. F. Ervin, M. Wagner, H. A. Gordon, T. D. Luckey, R. A. Brown, G. J. Mannering, and C. J. Campbell: Germ-Free Chicken Nutrition I. Gross Development and Vitamin Utilization Studies Employing White Leghorn Chicks. *J. Nutr.*, 41: 31–49, 1950.

Reyniers, J. A., P. C. Trexler, R. F. Ervin, M. Wagner, T. D. Luckey, and H. A. Gordon: Rearing Germ-Free Chickens. *Lob. Rept.*, 2: 1–116, 1949.

Reyniers, J. A., P. C. Trexler, R. F. Ervin, M. Wagner, T. D. Luckey, and H. A. Gordon: Some Observations on Germ-Free Bantam Chickens. *Lob. Rept.*, 2: 119–148, 1949.

Reyniers, J. A., P. C. Trexler, R. F. Ervin, M. Wagner, T. D. Luckey, and H. A. Gordon: A Survey of Germfree Animals: 1. The White Wyandott Bantam and White Leghorn Chicken. *Lob. Rept.*, (in press).

Schottelius, Max: Die Bedeutung der Darmbakterien fur die Ernahrung I. *Arch. F. Hyg.*, 34: 210–243, 1899.

Schottelius, M.: Die Bedeutung der Darmbakterien fur die Ernahrung III. *Arch. F. Hyg.*, 67: 177–208, 1908.

Wagner, M.: Germfree Research. A Basic Study in Host-Contaminant Relationship II. Serologic Observations in Germfree animals. *Bull. N.Y. Acad. Med.*, 31: 236–239, 1955.

du Vigneaud, V., C. Reseler, J. R. Rachele, J. A. Reyniers, and T. D. Luckey: The Synthesis of 'Biologically Labile" Methyl Groups in the Germ-Free Rat. *J. Nutr.*, 45: 361–376, 1951.

PROGRESSIVE BIOCHEMICAL CHANGE IN THE LEUKEMIC MOUSE

Roy B. Mefferd, Jr.,
Southwest Foundation for Research and Education and
The University of Texas, Austin

Following the transplantation of strain homologous leukemia to mice there is an apparent latent period of about a week. During this period there is no gross change in the physiological characteristics of the animals. The work reported here had as its main object a study of chemical changes which might occur during this latent period.

For convenience in presentation, the work is divided into two parts—changes in excretion patterns, and changes in the rate of uptake of C^{14}-labelled acetate into several lipids at periods during the development of the leukemia, and following injection of non-leukemic species-homologous tissue, or of strain heterologous leukemia.

Excretion patterns of 3-4 week-old male C57B1/10 and C57B1/10-H-2^d mice grouped by litters into units of 6 mice each, and of their F1 and backcross generations were determined 24 hours prior to transplantation of myeloid leukemia C-1948, and 3, 7, 10, 15, and 30 days thereafter. Complete 24-hour fasting urine specimens collected at each time interval indicated, were analyzed in triplicate for a number of nitrogenous and mineral substances.

The C57/B1/10 animals are susceptible to C-1498 and all died between the 12th and 15th days, while none of the resistant C57B1/10-H-2^d mice died. The tumors in the latter animals developed progressively until about the 8th day, when they began to regress. On day-30 scabs over the original site of the tumor were still evident.

The strains were identical in their response with respect to a number of the compounds, but were different with respect to others. This represents a chemical difference traceable to a single gene change. By day-3 many of the compounds were excreted in

significantly elevated quantities, and many of these changes in excretion persisted throughout the remainder of the period.

Lipid metabolism was found to follow about the same pattern as the excretion data in the sense that changes were detectable very early following transplantation. Carboxyl-labelled acetate was injected i.p. into mice at various time intervals following transplantation to either strain homologous or heterologous leukemia, or of various strain homologous organ macerates, i.e., liver, kidney, spleen. Two hours later the animals were sacrificed by a blow on the head and immediately were dissolved in 33% KOH to stop metabolic activity. Following hydrolysis total non-saponifiable lipids, cholesterol, total fatty acids, saturated and unsaturated fatty acids were fractionated and their specific activity determined. In some of the experiments, the digitonide-precipitated cholesterol was purified further via the dibromide to permit determination of the so-called higher counting substances which are precipitated as the digitonide with cholesterol. Specific activities were determined from end-window counts corrected for self-absorption, and some samples were also counted by means of a scintillation counter.

DBA-2, Euer (Webster derived) and BAF-1 3-4 week old males were injected with either lymphoid leukemia, P-1534, myeloid leukemia, C-1498, or various organ macerates.

A 24-hour fast prior injection of the acetate markedly depressed its rate of incorporation into all lipids examined. Injection of any substance other than saline modified the rates, and the changes which occurred as homologous leukemia developed followed a rather characteristic pattern. For example, the rate of incorporation into cholesterol distinctly increased within a few hours of transplantation. Likewise there were very early and significant alterations in the higher counting substances.

It is evident that chemical changes occur very shortly after transplantation of homologous leukemia into mice. Some of these are due to the stress of injection and the reaction to "foreign" tissue, but certain of these chemical changes appear to be distinctive to the development of strain homologous leukemia.

CARCINOGENESIS IN THE WHITE PEKIN DUCK*

R. H. Rigdon

Both spontaneous and induced tumors have been reported infrequently in the duck (Historical references). No report of an experimentally induced skin tumor in white Pekin ducks has been found in the literature. During a period of 15 years a large number of this particular strain of ducks has been used in our laboratory and no spontaneous tumor has been observed. Recently methylcholanthrene has been used to induce tumors in this host. The following types of tumors have occurred in the skin after local applications of methylcholanthrene: papillomas, hemangiomas, fibromas, squamous cell carcinomas, neurofibromas, ganglioneuromas and Pacinian corpuscle tumors (Rigdon, 1952, 1953, 1954, 1955a, 1955b). A ganglioneuroma and a hemangioma have been observed to develop in the pectoral muscle following the implantation of methylcholanthrene crystals (Rigdon, 1955b) and the injection of methylcholanthrene in sesame oil (Rigdon, 1952). In all instances tumors have occurred either in the area of tissue treated or in areas of skin contaminated by this carcinogen while the ducks preened. Tumors resulting from the latter usually were located on the head and feet. No metastatic tumors have been found in the duck. The majority of the tumors were typical benign lesions; however, invasion of the pectoral muscle did occur in the locally induced hemangiomas (Rigdon, 1952). The infiltration of the stroma by squamous epithelial cells with subsequent spontaneous regression of the lesion has presented a problem in nomenclature.

A 0.25 per cent solution of methylcholanthrene in acetone has been the preparation used for topical applications. The birds usually varied in age from 10 to 50 days at the time the carcinogen was first applied. The maximum time of observation has been two years. In some experiments, following a surgical incision

* From Laboratory of Experimental Pathology, The University of Texas, Medical Branch, Galveston, Texas. This study was aided by several grants C-1469 (3C) from the National Cancer Institute of the National Institutes of Health, Public Health Service, and the Tobacco Industry Research Committee. Presented at the M. D. Anderson Symposium March 31, 1956.

through the skin and muscle, 50 or 75 mg. of methylcholanthrene crystals were put directly into the pectoral muscle and the area closed with sutures. This carcinogen also has been suspended in both sesame and mineral oil for subcutaneous, intramuscular and intratracheal injections. All suspensions have been unsatisfactory for a quantitative study since the crystals did not remain uniformly suspended. Pellets, 5 x 2 mm. in size and approximately 5 mg. in weight, have been made by firmly compressing the methylcholanthrene crystals (Omer, 1954). Crystals (50 mg.) have been mixed with flour and put into gelatin capsules, size number 1, for oral administration.

The variation in the response of the duck to the above carcinogenic preparations has been an interesting feature of this study. The number of daily topical applications of methylcholanthrene in acetone for maximum tumor response would appear to be approximately thirty (Rigdon, Trauma and Cancer). Only a few observations have been made referable to the effect of a second or third series of applications of the same carcinogen to the same area of skin. However, in the few birds in which such a procedure has been carried out, the local tumor response did not appear to be very different following the second series, when compared with the first made several months previously. Obviously, in such a study, the age of the duck must be carefully controlled.

The topical applications of methylcholanthrene in acetone usually have been made to the skin of the body beneath the wing and to the skin on the under surface of the corresponding wing. An attempt was made to thoroughly moisten the area by dropping the preparation from either a pipette or hypodermic needle onto the skin. The amount necessary to cover the area varied from 0.2 to 2.0 ml., depending on the size of the bird. In our experiments greater attention has been given to producing tumors than to any quantitative study. Desquamation and ulceration in the treated areas of skin frequently occurred following local applications of the methylcholanthrene. Sometimes it was necessary to discontinue all treatments to permit healing. There does not appear to be any relation between the frequency of skin tumors and the degree of ulceration produced by methylcholanthrene. In fact, it is our impression at this time that skin tumors are likely to be more numerous in those ducks where ulceration does not occur.

The presence and absence of feathers must be carefully evaluated in these studies. In many of the experiments the feathers were plucked once per week, both during the time the carcinogen was being applied and thereafter during the entire period of the experiment. In other experiments, the feathers were plucked for the first time after all applications of methylcholanthrene were discontinued. The variation in tumor response of birds with feathers plucked and unplucked has been reported (Rigdon, Trauma and Cancer). It may be stated at this time that a larger number of papillomas and hemangiomas has occurred in ducks where this carcinogen was applied and the feathers were subsequently plucked than in birds in which the feathers were not plucked. It has been suggested in these experiments that trauma may have acted as a co-carcinogen following the plucking of feathers (Rigdon, Trauma and Cancer).

The time of occurrence of the different tumors has varied following local applications of this carcinogen. Papillomas (Fig. 1) and hemangiomas (Figs. 2 and 3) were the first tumors to occur

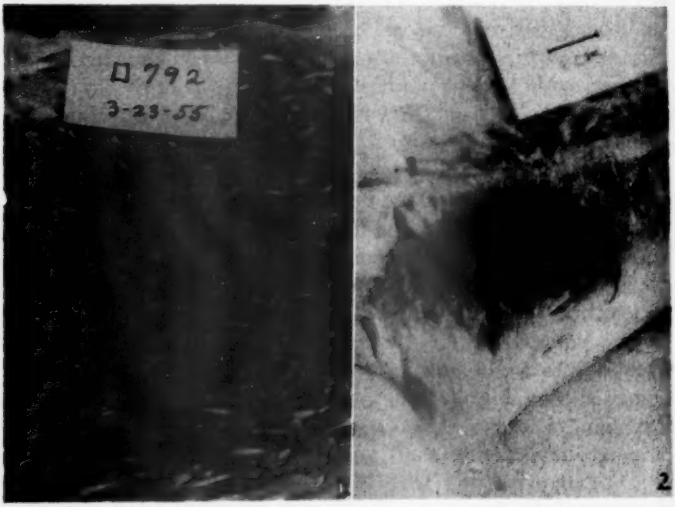


FIG. 1. Duck 792. Methylcholanthrene in acetone was applied daily for 30 days to the skin. This photograph made 40 days after the last application of the carcinogen. The papillomas are too numerous to count.

FIG. 2. Duck 462. Methylcholanthrene applied to the skin 30 times. This tumor photographed 15 months after the last application of the carcinogen.

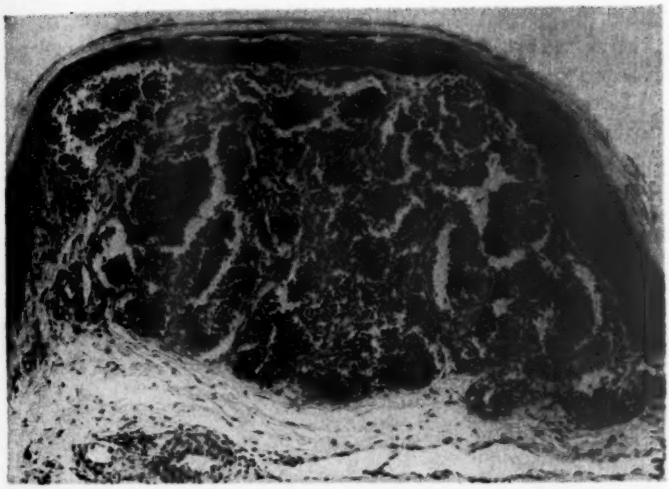


FIG. 3, Duck 781. This is a hemangioma that occurred on the web of the foot. There is a wide variation in the size of the vascular channels in different and in the same tumor. H and E X 140.

in the skin of the duck. They usually appeared within 30 to 60 days after the last application of the acetone solution of methylcholanthrene. Papillomas continued to develop for two to three months, while the hemangiomas continued to appear for as long as a year. Papillomas and hemangiomas were the most frequent types of tumor to occur in the skin. The number in a single bird varied from one to 50. They occurred in 90 per cent or more of our treated ducks. The other types of tumors observed in the skin were much slower in making their appearance and no specific time interval can be given for their appearance.

Observations were made on the variation in tumor response of different areas of skin to the same preparation of methylcholanthrene (Rigdon, Tumors Induced in Skin Without Follicles). These areas were (1) the skin of the body wall, (2) the skin beneath the lower bill in the areas devoid of feathers, and (3) the web of the foot. Each type of tumor previously referred to occurred in the skin of the body wall and wing. Only papillomas occurred in the skin beneath the bill, while papillomas and hemangiomas were frequently found on the web of the foot. Only one neurofibroma was observed to develop in the web. Obviously, one

could not predict the type of tumor which would occur in the skin on one area of the body after one had applied the same carcinogen to the skin in another area.

Tumors developed only in the area of the body where the carcinogen was applied. Ducks preened after the methylcholanthrene in acetone was put on the skin of the body wall, thus frequently contaminating the skin about the head and the web of the feet. Many tumors developed in these latter sites as a result of this contamination (Fig. 4). No tumor was observed to metastasize in these experiments.

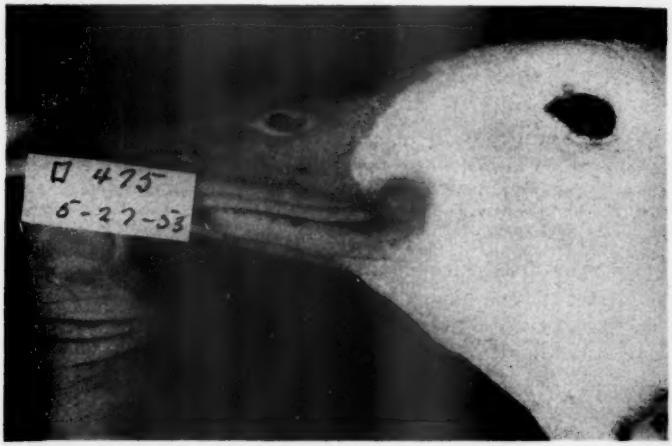


FIG. 4, Duck 475. Methylcholanthrene in acetone was applied daily for 30 days to the skin beneath the left wing. The head was contaminated with the carcinogen while the duck preened. Note the hemangioma at the angle of the mouth and the papilloma in the skin beneath the bill. This photograph was made 127 days after the last application of the carcinogen.

The response of the tissues of the duck to methylcholanthrene pellets is interesting (Omer, 1954). They were put into the brain, pectoral muscle and subcutaneous tissues. For injection the pellets were put into a 16 gauge needle and pushed into the tissue by a plunger. One pellet was put into the cranial cavity of each of 48 ducks; most of these were put into the brain tissue, while a few were left in the subarachnoid space about the base of the brain. Only a few of the birds died within the first ten days. Twenty ducks were observed for 20 to 99 days, 10 from 100 to 299 days,

and 9 for 300+ days. Few of the ducks showed any abnormal neurogenic manifestations following the injection of the pellets.

When the ducks were sacrificed, the pellets were demonstrable macroscopically and looked the same as when they were injected (Fig. 5). There was only a small amount of histologic reaction about the pellets. There was nothing to suggest a new growth in any bird. Likewise, pellets when injected into the muscle of 23 ducks did not produce any significant reaction and no tumors occurred (Fig. 6). Pellets injected into the subcutaneous tissue of 23 ducks apparently were dissolved within an interval of 30 to 60 days. No tumors developed where these pellets were injected.

The failure of tumors to develop and the absence of a macroscopic reaction in the pectoral muscle following the injection of pellets is of interest in view of the results obtained when methylcholanthrene crystals were placed directly into the same muscle.

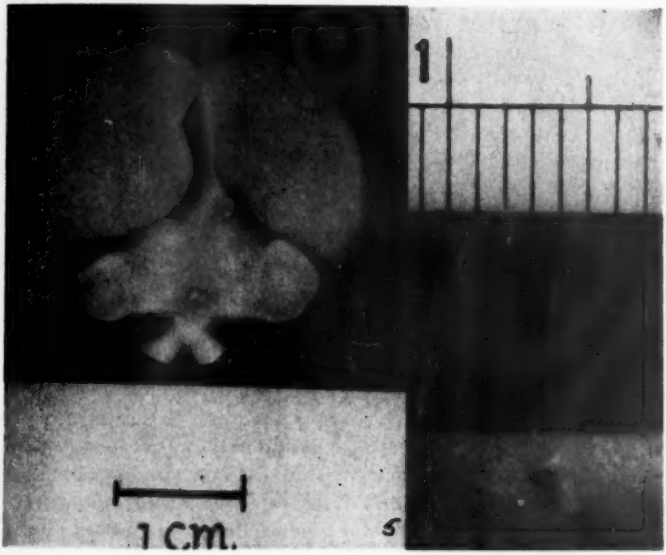


FIG. 5, Duck 661. This pellet of methylcholanthrene was injected into the brain 107 days before this duck was sacrificed. There is no macroscopic reaction around this pellet.

FIG. 6, Duck 921. This pellet of methylcholanthrene was injected into the pectoral muscle 338 days before this duck was sacrificed. The pellet is approximately the same size as when injected.

Either 50 or 75 mg. of methylcholanthrene crystals were put directly into the pectoral muscle of 33 ducks, varying in age from 20 to 82 days. Two of these (D 343 and 344) developed a ganglioneuroma and one (D 113), a hemangioma. The tumor in one of these (D 343) measured 2.5 cms. in diameter on the 180th experimental day, at which time a biopsy was removed. The tumor continued to grow and measured 5 cms. when the bird was sacrificed on the 310th experimental day. At this time the tumor was surrounded by a zone of myxomatous tissue. It was firm and white. Histologically it was a typical ganglioneuroma (Figs. 7 and 8). A considerable acute and chronic reaction was present about the methylcholanthrene crystals in ducks sacrificed between the 10th and 100th day. This reaction was extensive after 30 days. In the ducks that were kept for 200 days or longer it was difficult in some cases to say whether the dense fibrous tissue represented an inflammatory or a neoplastic response.

A variety of neurogenic tumors occurred in the skins of ducks following local applications of methylcholanthrene (Rigdon, 1955b). These included neurofibromas (Fig. 9), ganglioneuromas, and Pacinian corpuscle tumors. The earliest appearance of a tumor of this group was on the 50th experimental day; they continued to appear in the skin until the 278th day. Some of the ducks with these neurogenic tumors were observed until the 427th experimental day. The size of these tumors varied from 0.5 to 4.0 cms. A characteristic feature of these lesions was the presence of multiple types of nerve tissue in a single tumor. Typical tactile corpuscles and ganglion cells were present in the ganglioneuroma occurring in the pectoral muscle of D 343 (Figs. 7 and 8). Many tactile-like corpuscles were found in other tumors in association with adult nerve cells. In the Pacinian corpuscle tumors ganglion cells were surrounded by a capsule similar to that around the ganglion cells in the sympathetic nervous system. The multiplicity of cell types within a single tumor would suggest that some cell with potentiality to develop into a tactile corpuscle, an adult nerve cell, and a neuroblast was affected by the methylcholanthrene.

Another solid tumor observed in the skin of these ducks treated locally with methylcholanthrene was a fibroma (Figs. 10 and 11). These tumors usually were less than 2 cm. in diameter. Some were firm and others were relatively soft. Histologically they

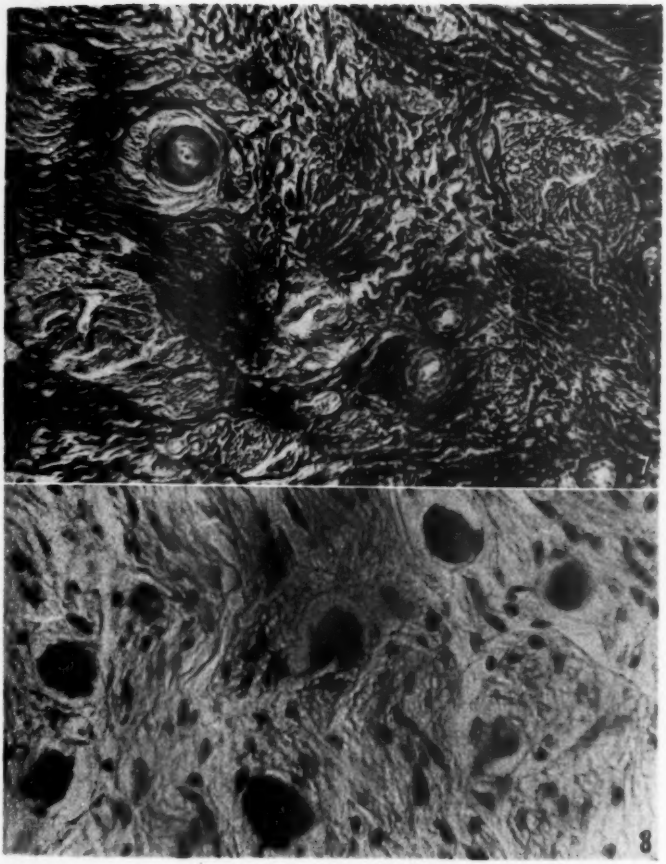


FIG. 7, Duck 343. Fifty mg. of methylcholanthrene were put into the pectoral muscle 278 days previously. Note the interlacing bundles of collagen and nerves. This is a typical ganglioneuroma with a Pacinian corpuscle in the tumor. Del Rio Hortega's stain, X 170.

FIG. 8, Duck 343. Same as figure 7. This shows ganglion cells in the same tumor. Bodain stain X 516.

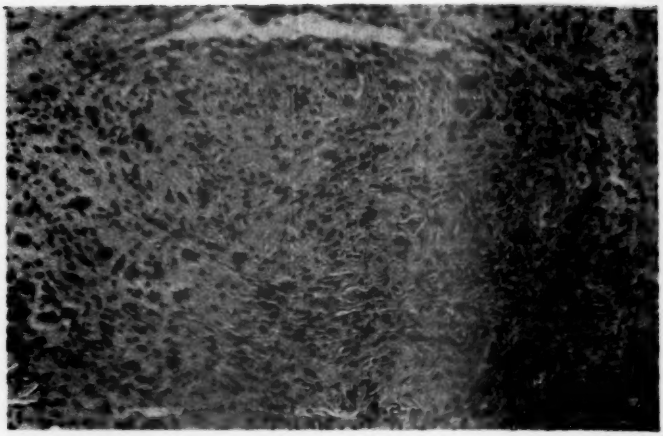


FIG. 9. Duck 461. This shows the pallasading of cells and the variation in size and shape of the cells so characteristic of these neurofibromas. H and E X 156.

were typical of this type of lesion. Some showed groups of lymphocytes within the tumor tissue. Some of these fibromas arose from a feather follicle (Rigdon, 1953). Six ducks showed this particular lesion. The largest tumor was 1.0 cm. in diameter. They were attached by a narrow pedicle. Because of this, the blood supply no doubt was poor, which may have accounted for their spontaneous disappearance.

Fat was a conspicuous feature in the regressing hemangiomas; however, we found only one specific lipoma (D 455). This measured 1.5 cm. in diameter (Fig. 12). On section it was lobulated, yellow, and relatively soft. Microscopically it was a typical lipoma with little fibrous stroma (Fig. 13). This lipoma occurred in the skin beneath the left wing of a duck that was treated locally on the right side of the body. Because of this and since similar tumors have not occurred in methylcholanthrene-treated areas of other ducks, it is likely that this lipoma was a spontaneous tumor and not related in any way to the carcinogen.

Papillomas were the most frequent type of tumor to occur in the skin of the body wall following local application of methylcholanthrene in acetone. It was the only tumor to occur in the skin of the lower bill (Rigdon, Tumors Induced in Skin Without

Follicles). Very few papillomas developed on the web of the foot (Rigdon, Tumors Induced in Skin Without Follicles). Some of these on the skin of the body measured 5 to 9 mm. in height and as much as 2 cm. in diameter. Frequently these papillomas were so numerous on the skin that they could not be counted (Fig. 1). All papillomas spontaneously disappeared.

A papilloma was removed from the skin of twenty ducks, 26 days after the last application of methylcholanthrene. One-half of the lesion was fixed in formalin for histologic study and the other

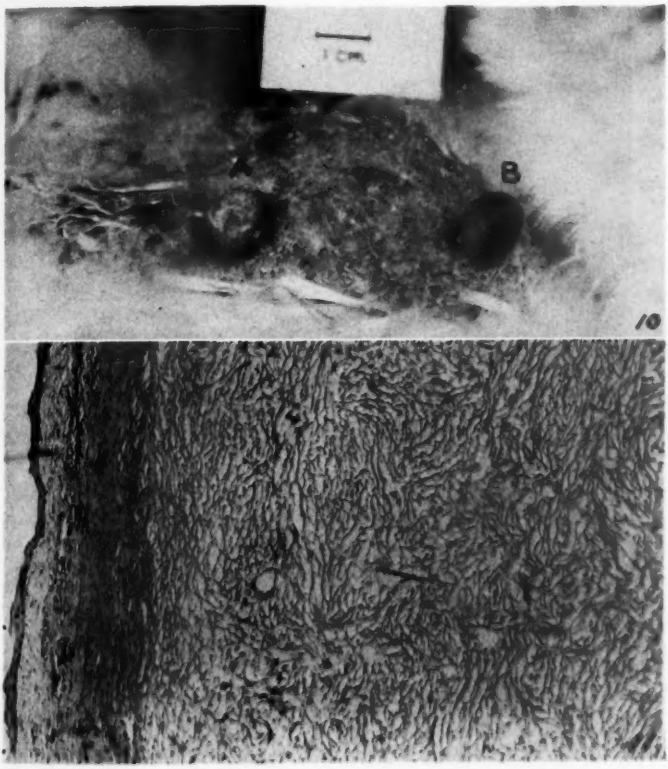


FIG. 10, Duck 462. Same as shown in figure 2: A-Fibroma B-Hemangioma.

FIG. 11, Duck 462. Microscopic section of the fibroma shown in figure 10-A., H and E X 110.

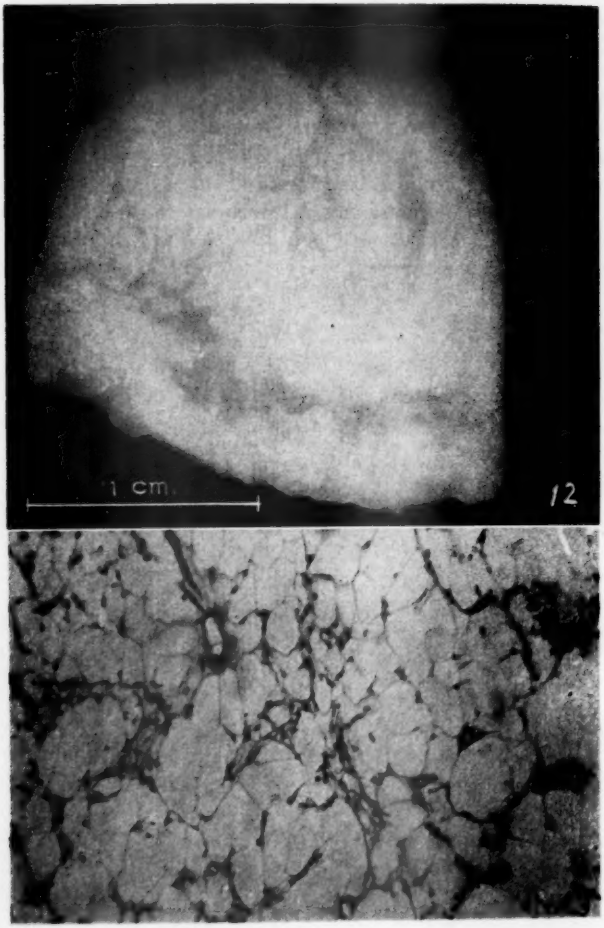


FIG. 12. Duck 455. This lipoma occurred in the skin on the left side of the duck and the methylcholanthrene was put on the right side. On section it was lobulated, yellow, and relatively soft.

FIG. 13. Duck 455. Same as figure 12. A typical lipoma. H and E X 102.

was used to inoculate the chorio-allantoic membrane of developing chick embryos. All cultures were negative. Such results obviously do not support the opinion expressed by Duran-Reynals (1951–52) that papillomas in the chicken result from an activation of fowl pox virus by this carcinogen. It would appear more likely that the papillomas occurring in the duck are only a manifestation of the direct effect of this carcinogen on the dermis. Should one conclude that a virus is the cause of these papillomas, then the question would naturally arise as to whether the same or a different virus was stimulated by the methylcholanthrene to produce each of the several different types of tumors that have occurred in the duck. As far as we know the virus of fowl pox has not been grown in the duck.

Squamous cell carcinomas occurred in the skin of the duck following the local application of methylcholanthrene (Rigdon, 1952). It was difficult to determine the frequency of this lesion since many resembled a papilloma, while others were superficial ulcerations. To be sure of the diagnosis, it was necessary to remove the lesion for histologic study. Squamous cell carcinomas were observed as early as the 63rd experimental day (D 240) and as late as the 283rd day (D 145). Some may wish to refer to these lesions as "carcinomatoid" tumors since they spontaneously regressed and no metastases occurred. There was an infiltration of epithelial cells down into the stroma with intercellular bridges, mitotic figures and epithelial pearls (Figs. 14, 15, and 16). A similar squamous cell tumor has been produced in the chicken (Rigdon and Brashear, 1954; Rigdon and Hooks). The development and regression of this type of lesion has been studied more extensively in the chicken than in the duck. It has been suggested that "the absence of a lymphatic system in the skin of fowls, like that in mammals, may be a significant factor affecting the development, regression, and spread of malignant tumors" (Rigdon and Brashear, 1954).

As previously stated, a hemangioma was one of the most frequent tumors to occur in the skin following local applications of methylcholanthrene in acetone. They varied in size from a few millimeters to five centimeters. They showed the characteristic features of either a capillary or cavernous type of hemangioma, or a combination of these. Two ducks from a group of 19, injected intramuscularly with methylcholanthrene in mineral oil (10

mg./cc.), developed a hemangioma in the pectoral muscle. No line of demarcation separated the tumor from the surrounding muscle (Fig. 17). The tumor tissue was grayish-white and gelatinous. There were areas in which the neoplasm was formed by small endothelium-lined sinuses, some of which infiltrated the muscle (Figs. 18 and 19). Fat, fibrous tissue, mucoid material, and collections of lymphocytes were present in the old regressing hemangiomas.

The vascular channels comprising these hemangiomas were studied by injecting red and blue latex into the arterial and venous systems (Rigdon, 1953) (Fig. 20). The blue and red latex was found within the same vascular channel, indicating a mixing of arterial and venous blood. Recently it has been suggested that the histiocytes normally present in the skin were stimulated by methylcholanthrene to form angioblasts (Rigdon, *et al.*). These cells proliferate, form channels, and subsequently communicate with vessels arising from normal arteries and veins in the local areas to form these hemangiomas. One of the most interesting features of these hemangiomas has been their spontaneous regression. The mechanism by which this regression occurs has been discussed in previous publications (Rigdon, 1955a; Rigdon, *et al.*). It may suffice at this time to state that the regression results from a local proliferation of lymphocytes within the walls of these vascular channels, resulting in stasis of the circulating blood; subsequently these occluded channels are replaced by collagen and fat.

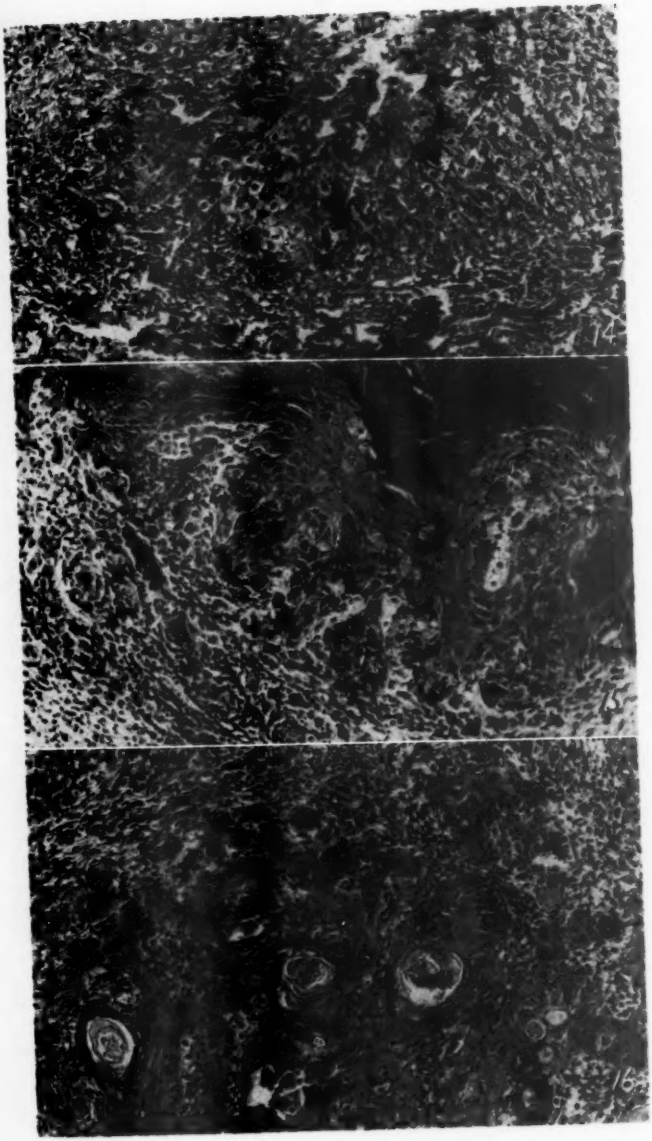
Attempts are now being made to produce tumors in the respiratory tract by injecting methylcholanthrene in mineral oil directly into the trachea through the larynx. This is accomplished by the use of a plastic tube on the end of a small syringe. One-half

---

FIG. 14, Duck 143. Skin treated 30 times with methylcholanthrene. A small ulcerated area was removed 3 days after the last treatment. A wide zone of squamous epithelial cells form the base of this ulcer. H and E X 190.

FIG. 15, Duck 407. This was a small papilloma covered by a thick zone of keratin. The base is formed by a zone of squamous cells that infiltrate the stroma. H and E. X. 190.

FIG. 16, Duck 240. On the 54th day following the last application of methylcholanthrene to the skin there was a small papilloma. The base measured 3 mm. and was granular. The lesion was removed. Note the squamous cells that form the base of this ulcer. It has all the features of a typical squamous cell carcinoma. H and E X 190.



ml. of mineral oil containing 15 mg. of methylcholanthrene per ml. is injected daily. After 15 to 20 injections a few of the ducks have shown signs of tracheal obstruction (Fig. 21). Treatments were temporarily discontinued but were resumed two to three

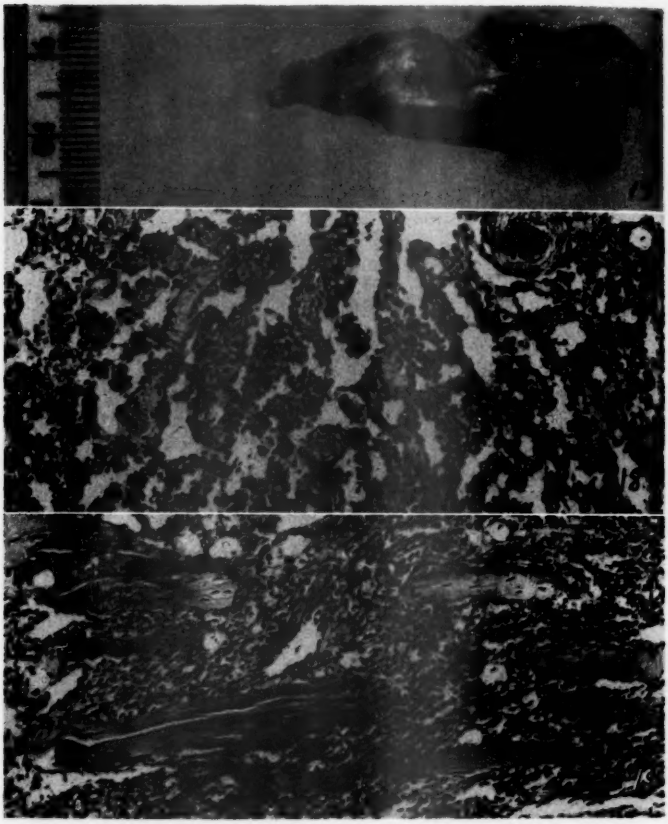


FIG. 17, Duck 113. Hemangioma in pectoral muscle. Methylcholanthrene in mineral oil was injected here 581 days previously. There is no sharp line of demarcation between the tumor and the surrounding muscle.

FIG. 18, Duck 113. The gross lesion is shown in figure 17. This shows the microscopic characteristics of this hemangioma H and E X 190.

FIG. 19, Duck 5. This hemangioma occurred in the pectoral muscle. Note the infiltration of the muscle by the tumor tissue. This is a characteristic finding in the hemangiomas that occurred in this muscle. H and E X 190.

weeks later. Some of the birds were sacrificed after they developed obstruction; the trachea in these birds showed a zone 3 to 6 cm. in length filled with a fibrinous exudate. No tumors have been observed; however, a larger group of ducks are now under observation. Methylcholanthrene crystals were blown from a 5 ml. pipette with the aid of a large rubber bulb into the trachea of a second group of ducks. The crystals were subsequently demonstrated by ultra-violet light in the proximal 4 cm. of the trachea.

FIG. 20, Duck 778. Hemangioma injected with red and blue latex. There is a wide variation in the size of the vascular channels that make up these tumors. There are many communications between the channels and a mixing of the two colors of latex within some of the larger spaces.

FIG. 21, Duck 1 103. Trachea with portion of attached lungs. Note the large amount of acute exudate in the distal half of the trachea; 0.5 ml. methylcholanthrene in mineral oil (15 mg./cc.) put into trachea for 17 days. Respiratory difficulty occurred at this time and the bird was sacrificed.

Four of these ducks have been observed for one year and no tumors have developed.

The neoplastic response of the gastro-intestinal tract of the duck to daily oral administration of methylcholanthrene in gelatin capsules is being studied. Preliminary observations indicated that 50 mg. of methylcholanthrene may be given twice a day to adult ducks of 12 days without producing any clinical manifestation of toxicity. The tumor response to methylcholanthrene in both the respiratory and intestinal tracts will be compared with that in the skin of the duck.

All attempts to transplant these hemangiomas and ganglioneuromas have failed; however, further attempts will be made to grow these tumors on the chorio-allantoic membrane and in the yolk sac of the chick embryo.

In summary, it may be said that the white Pekin duck is a good host for the study of tumors produced by methylcholanthrene. As far as we know, this is the first time a ganglioneuroma and a Pacinian corpuscle tumor have been experimentally produced. A tumor also has been produced with the morphological characteristics of a squamous cell carcinoma that spontaneously regresses. This lesion would seem to be an excellent one for studying certain characteristics of malignant change. It may be that the absence of a lymphatic system in the duck, such as we have in mammals, is a significant factor in the mechanism of the spread of tumors. Regression of hemangiomas in the duck also is an interesting phenomenon in carcinogenesis. Time does not permit a discussion of these lesions occurring in the duck referable to similar ones observed by others and recorded in the literature.

REFERENCES

Alezois and Cotte, J.: Tumeur du médiastin à tissue multiples chez un canard. *C. rend. Soc. Biol.*, 64: 525–526, 1908.

Babić, Ivo: Spontani tumori peradi. *Veterinarski Arhiv.*, 1: 158–250, 1931.

Bulliard, H., and Ch. Champy: Etude d'un embryome du canard envahi secondairement par une tumeur maligne: neuro-épitheliome. *Bull. A. France, Etude Cancer*, 20: 197–205, 1931.

Campbell, J. G.: Primary carcinoma of the liver in the duck. *Nature*, Lond., 158: 711–712, 1946.

Campbell, J. G.: Spontaneous hepatocellular and cholangiocellular carcinoma in the duck. *Brit. J. Cancer*, 3: 198–210, 1949.

Dougherty, E. III: Disease Problems confronting the duck industry. Proceeding book. American Veterinary Medical Association, Nineteenth Annual Meeting. Toronto, July 20–23, 1953, pp. 359–365.

Duran-Reynals, F.: Age susceptibility of ducks to the virus of the Rous sarcoma and variation of the virus in the duck. *Science*, 193: 501–502, 1941.

Duran-Reynals, F.: The significance of the non-neoplastic lesions induced in the central nervous system of ducklings by the virus of a duck variant of the Rous sarcoma. *Yale J. Biol. and Med.*, 22: 555–564, 1950.

Duran-Reynals, F.: A Study of 3 New Duck Variants of the Rous Chicken Sarcoma. *Cancer Res.*, 7: 99–102, 1947.

Duran-Reynals, F.: Studies on the Combined Effects of Fowl Pox Virus and Methylcholanthrene in Chickens. *Ann. New York Acad. Sci.*, 54: 977–991, 1951–52.

Duran-Reynals, F.; E. W. Shrigley, and E. De Hostos: Carcinogenesis Induced by Methylcholanthrene in Pigeons, Guinea Fowls, and Ducks. *Cancer Res.*, 5: 11–17, 1945.

Eber, August, and Erich Malke: Geschwulstbildungen beim Hausgeflügel Zusammenfassender. Bericht über 392 bei 16460 Geflügelsektionen ermittelte Neubildungen (Tumors in Domesticated Birds). *Zeitschrift f. Krebsforschung*, 36: 178–192, 1932.

Fujinami, A., and S. Hatano: Contribution of Pathology of Heterotransplantation of Tumor. A Duck Sarcoma from Chicken Sarcoma. *Gann.*, 23: 67–75, 1929.

Gye, W. E.: A note on the propagation of Fujinami's fowl myxo-sarcoma in ducks. *Brit. J. Exp. Path.*, 12: 93–97, 1931.

Hook, Heinrich: Zur Kasuistik der Geschwulste bei Enten (Ein Carcinom) (Tumors in ducks. A case of carcinoma). *Ztschr. f. Krebsforsch*, 23: 482–487, 1926.

Joest, E., and S. Ernesti: Untersuchungen über spontane Geschwülste bei Vögeln. *Zschr. Krebsforsch*, 15: 1–75, 1915.

Lombard, Charles: Volumineux tératome de la cavité abdominale chez le canard. *Rev. Vet. et J. Med. Vet. Toulouse*, 87: 630–632, 1935.

Muorohara, N.: The relation between the heterotransplantation of tumor and the internal secretion. Implantation of chicken sarcoma into the duck and the function of the thyroid gland. *Trans. Jap. Pathology Soc.*, 21: 780–785, 1931.

Omer, Syed: Effects of Methylcholanthrene on Brain of Ducks. Thesis for Degree of Master of Arts in Pathology, The University of Texas. 1954.

Purdy, W. J.: Fujinami myxsarcoma in ducks: resistance to reinfection. *Brit. J. Exper. Path.*, 14: 297–302, 1933. Abst: *Am. J. Cancer*, 22: 914, 1934.

Purdy, W. J.: The propagation of the Rous Sarcoma No. 1 in ducklings. *Brit. J. Exptl. Path.*, 13: 473–479, 1932.

Ratcliffe, Herbert L.: Incidence and nature of tumors in captive wild mammals and birds. *Am. J. Cancer*, 17: 116–135, 1933.

Rigdon, R. H.: Tumors Produced by Methylcholanthrene in the Duck. Papillomas, Squamous Cell Carcinomas and Hemangiomas. *A.M.A. Arch. Path.*, 54: 368–377, 1952.

Rigdon, R. H.: Fibroma Arising from Feather Follicles of Ducks following Local Application of Methylcholanthrene to Skin. *Proc. Soc. Experi. Biol. and Med.*, 83: 34–36, 1953.

Rigdon, R. H.: Spontaneous Regression of Neoplasms. An Experimental Study in the Duck. *Southern Med. J.*, 47: 303–310, 1954.

Rigdon, R. H.: Spontaneous Regression of Hemangiomas. An Experimental Study in the Duck and Chicken. *Cancer Research*, 15: 77–79, 1955.

Rigdon, R. H.: Neurogenic Tumors Produced by Methylcholanthrene in the White Pekin Duck. *Cancer*, 8: 906–915, 1955.

Rigdon, R. H.: Trauma and Cancer. An Experimental Study in the Duck. *A.M.A. Arch. Path.*, 61: 443–449, 1956.

Rigdon, R. H.: Tumors Induced in Skin without Follicles. An Experimental Study in the Duck, to be published.

Rigdon, R. H., and Doyle Brashear: Experimental Production of Squamous-Cell Carcinomas in the Skin of Chickens. *Cancer Research*, 14: 629–631, 1954.

Rigdon, R. H., and Murray D. Hooks: A Consideration of the Mechanism by which Squamous Cell Carcinomatoid Tumors in the Chicken Spontaneously Regress. *Cancer Research*, 16: 246–250, 1956.

Rigdon, R. H., Jack Walker, and A. H. Teddlie: Hemangiomas. An Experimental Study in the Duck, to be published.

Saijo, Yoshika: Studies on the pathologic change of the spleen of sarcoma ducks. *Trans. Jap. Path. Soc.*, 19: 626–633, 1929.

Sumikoski, Yoshio: Biological studies of duck sarcoma. *Trans. Jap. Path. Soc.*, 19: 655–56, 1929.

Urbain-Leblanc, Prix: L'élevage industriel Canards chinois en Cochinchine et sa pathologie. *Bull. Acad. Vet. France*, 9: 181–189, 1936.

Von Christiane Tamaschke: Beitrage zur nergleichenden oukologie der Haussaugetiere. Wissenschaftliche Zeitschrift der Humboldt. Universitat Berlin, January 1, 1951.

Worms, G., and H. P. Klotz: Contribution à l'étude des tumeurs thymiques. A propos d'un cas d'épithéliome thymique chez un canard. *Bull. A. France, Etude Cancer*, 23: 420–432, 1934.

Yoshido, N., and K. Kamoto: Studies on the primary hepatomas in ducks. *Jap. J. Vet. Sci.*, Vol. II, 35–52, 1949. (Abstract the *Vet. Bull.*, 22: 402, 1952 No. 2144).

PRECANCEROUS METABOLIC ALTERATIONS IN THE PROCESS OF AZO DYE CARCINOGENESIS*

J. D. SPAIN†

This is a report of some of the work which was carried out under the direction of Doctor Griffin while still at Stanford University and is being continued at M. D. Anderson Hospital. We will be concerned mainly with the early phases of the process of cancer induction by 3'methyl-4-dimethylaminoazobenzene (3'MeDAB). The compound produces a variety of liver tumors when fed to the albino rat at the level of 0.06 per cent in a semi-synthetic maintenance diet. These include malignant hepatomas, adenocarcinoma of the liver cell type and adenocarcinoma of the bile duct type (11). Ordinarily, the treatment which is employed in our laboratory can be expected to produce 100 per cent tumor incidence in approximately twelve weeks.

Cancer production, however, is preceded by a series of morphological changes, the most striking of which is the so-called bile-duct cell proliferation. In many cases the bile-duct cells increase by 100 to 300 fold during the period of 15 to 40 days following the start of continuous azo dye feeding (10, 13). This proliferation assumes more importance when it is realized that most of the tumors appear to arise from these hyperplastic areas. However, this proliferation plays a different role when interpreting biochemical data obtained during this phase of the process. Since most biochemical studies have been carried out on the livers of rats which had been treated with azo dyes for a least three weeks, many of the changes observed may be considered as the result of "diluting" parenchymal tissue with a tissue of entirely different character, that of bile duct cells. Such a view was adopted by Price, and the Millers at Wisconsin (10) and by the Schneider group at Bethesda (13) about 1950.

* From the Biochemistry Department, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas. This study was supported by funds from the Welch Foundation Grant.

† Present address: Michigan College of Mining and Technology, Houghton, Michigan. The author is indebted to Dr. A. C. Griffin for his guidance during the course of this work.

A preliminary study which illustrates this point was performed in our laboratory on succinic dehydrogenase activity in the livers of rats fed 3'MeDAB for varying periods of time up to 26 days. Liver homogenates were studied using the manometric procedure of Schneider and Potter (12) and the results were expressed as per cent of control values using the dry weight basis. As observed by Potter *et al.*, (9) the average enzyme activity was lowered by the treatment. However, when examined with respect to the extent of bile-duct cell proliferation, determined by the Chalkley counting procedure, (1), the results indicate that the alteration observed in succinic dehydrogenase activity was probably a reflection of changes in cell population. (Fig. 1.)

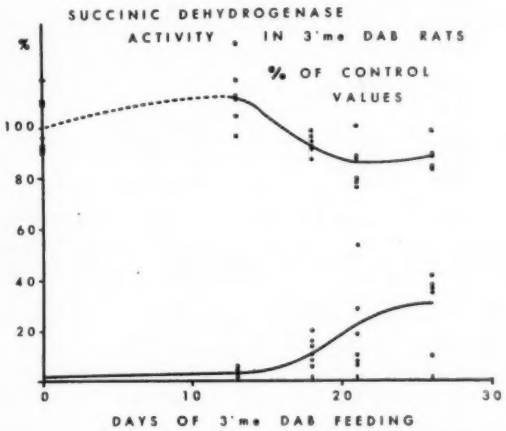


FIG. 1. Succinic dehydrogenase activity in the livers of rats fed 3'Me-DAB for various periods of time (upper curve) compared to the extent of bile duct proliferation (lower curve).

Another enzyme, glucose-6-phosphatase, which has been reported altered during 4-dimethylaminoazobenzene (DAB) cancer induction by Weber and Cantero (14), was examined in the same way following 3'MeDAB treatment using the method of Cori and Cori (3). In this case, a significant reduction in enzyme activity was observed prior to the onset of proliferation. This point was verified more recently in a group of ten rats which were fed 3'MeDAB for 14 days while a similar group of controls was

pair-fed with basal diet. As seen in table 1, a significant reduction in glucose-6-phosphatase activity was observed on both the wet and dry weight basis. Only one rat in the group (R-11) showed a proliferation of bile-duct cells and these amounted to less than five per cent of the tissue volume.

TABLE 1

Glucose-6-phosphatase Activity in the Livers of Rats after Fourteen Days Treatment with 3'Methyl-4-dimethylaminoazobenzene as Compared with Pair-fed Control Rats

	Experiment I* Rat No.	μg.P/mg./Hr. Dry weight	μg.P/ 100mg./Hr. Wet weight	Experiment II* Rat No.	μg.P/mg./Hr. Dry weight	μg.P/ 100mg./Hr. Wet weight
Basal	R-1	52.6	1,985	R-6	34.3	1,052
Fed	R-2	53.4	1,955	R-7	39.6	1,232
Control	R-3	57.1	2,030	R-8	38.4	1,226
Rats	R-4	39.8	1,488	R-9	44.6	1,388
	R-5	48.1	1,653	R-10	32.9	1,065
	Ave.	50.2±6.7	1,822	Ave.	38.0±4.6	1,193
3'Me-DAB	R-11	30.4	1,002	R-16	23.2	624
Fed	R-12	43.6	1,290	R-17	31.5	865
Rats	R-13	35.2	1,090	R-18	30.0	815
	R-14	28.8	917	R-19	34.5	964
	R-15	29.9	935	R-20	33.7	920
	Ave.	33.6±6.1	1,048	Ave.	30.6±4.5	838

* Slight differences in experimental conditions make it impossible to combine Experiments I and II. They have therefore been examined separately for statistical purposes.

Riboflavin was originally shown to be diminshed in the livers of rats fed DAB by Kensler *et al.* (6). The phenomenon was further studied and extended to other dyes by Griffin and Baumann (5). In our study, the livers of rats fed 3'MeDAB for various short periods of time were examined using the modified procedure of Conner and Straub (2). It was observed that riboflavin reduction was roughly a linear function of time for about the first 40 days. The early depletion of 3'MeDAB was shown even better by a pair-feeding experiment which was conducted for 14 days. The results indicate that riboflavin is significantly lowered by two weeks treatment with this azo dye although extensive bile-duct cell proliferation again was not observed to have taken place in any of the livers involved. (Table 2.)

In another investigation, glycogen storage was studied histochemically using the periodic acid-Schiff technique. From our experience, all animals which had been fed 3'MeDAB for at least

14 days showed sharply demarcated areas of centrolobular glycogen depletion which involved as much as 50 per cent of the total liver in some cases. All pair-fed control animals showed an even lobular distribution of liver glycogen, although the total staining capacity of the livers varied greatly. The glycogen-depleting effect of 3'MeDAB was observed to take place as early as 11 days in some animals and appeared to reach its maximum just before the onset of bile-duct proliferation. Glycogen storage was first observed to have been altered following rather toxic injections of DAB by Orr and Price (8). These investigators showed that the alteration could be demonstrated by classical chemical procedures and was not a manifestation of altered food intake. Such an experiment was also carried out by Graffi and his coworkers in Germany using 3'Me-DAB(4). The work I present here represents the first histochemical demonstration of such a change following the usual dye-feeding regimen.

TABLE 2

The Effect of Fourteen Days Treatment with 3'Methyl-4-dimethylaminoazobenzene on Liver Riboflavin Storage

Group I, Received Basal Diet		Group II, Received 3'Me-DAB Diet	
Rat No.	Hepatic Riboflavin μg./gm.	Rat No.	Hepatic Riboflavin μg./gm.
A-1	30.7	A-11	18.3
A-2	34.4	A-12	15.8
A-3	30.8	A-13	20.1
A-4	33.0	A-14	15.5
A-5	25.0	A-15	16.6
A-6	24.5	A-16	16.3
A-7	26.8	A-17	15.7
A-8	27.3	A-18	15.9
A-9	27.6	A-19	17.7
A-10	28.5	A-20	14.8
Ave.	29.9±3.31	Ave.	16.7±1.52

In conjunction with the histochemical study of glycogen, two other liver constituents were examined during this early period. The cytoplasmic basophilic material which is believed to be ribonucleic acid (RNA) was first shown by Opie (7) to be depleted from the liver lobule in somewhat the same fashion as glycogen, the lesion being predominantly centrolobular. Our experience using pyronin stain with ribonuclease digestion as a control has verified this and shown the time of occurrence to be similar

to that of the glycogen depletion. Mitochondria, which are somewhat related to the basophilic granules according to Opie, were not significantly altered during this period. The nature and localization of the change in glycogen and RNA storage suggests a general disfunction of parenchymal cells similar to that observed during the early phases of other types of liver damage. The fact that the central zone which is distal to the blood supply is first to be affected by the azo dye suggests that oxygen tension is involved either as a primary factor or as a potentiating agent for the effect of the dye.

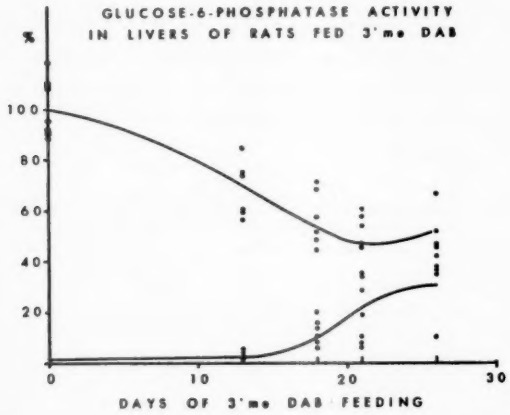


FIG. 2. Glucose-6-phosphatase activity in the livers of rats fed 3'Me-DAB for various periods of time (upper curve) compared to the extent of bile duct proliferation (lower curve).

In summation, it has been shown that alterations take place in at least four normal liver constituents well before and apparently independent of bile-duct cell proliferation. The results are interpreted as indicating a rather generalized degenerative process which may in turn be responsible for the subsequent morphological changes.

REFERENCES

1. Chalkley, H. W.: Method for Quantitative Morphological Analysis of Tissues. *J. Nat. Cancer Inst.*, 4: 47, 1953.
2. Conner, R. T., and C. J. Straub: Combined Determination of Riboflavin and Thiamin in Food Products. *Ind. Eng. Chem., Anal.*, Ed., 13: 385, 1941.

3. Cori, G. T., and C. F. Cori: Glucose-6-phosphatase of the Liver in Glycogen Storage Disease. *J. Biol. Chem.*, 199: 661, 1952.
4. Graffi, A., and W. Hebekerl: Uber chemishe Frühveranderungen der Rattenleber nach Verfütterung cancerogener azo Farbstoffe. *Arch. Geschwulstforsch.*, 5: 1, 1953.
5. Griffin, A. C., and C. A. Baumann: The Effect of Certain Azo Dyes upon the Storage of Riboflavin in the Liver. *Arch. Biochem.*, 11, 467, 1946.
6. Kensler, C. J., K. Sugiura, and C. P. Rhoads: Coenzyme I and Riboflavin Content of Livers of Rats Fed Butter Yellow. *Science*, 91: 623, 1940.
7. Opie, E. L.: Mobilization of Basophile Substance (RNA) in the Cytoplasm of Liver Cells with the Production of Tumors by Butter Yellow. *J. Exptl. Med.*, 84: 91, 1946.
8. Orr, J. W., and D. E. Price: Observations on the Hepatotoxic Action of the Carcinogen p-Dimethylaminoazobenzene. *J. Pathol. Bacteriol.*, 60: 461, 1948.
9. Potter, V. R., J. M. Price, E. C. Miller, and J. A. Miller: Studies of the Intracellular Composition of Livers from Rats Fed Various Amino Azo Dyes. III. Effects on Succinoxidase and Oxaloacetic Acid. *Cancer Res.*, 10: 28, 1950.
10. Price, J. M., J. W. Harman, E. C. Miller, and J. A. Miller: Progressive Microscopic Alterations in the Livers of Rats Fed the Hepatic Carcinogens 3'Methyl-4-dimethylaminoazobenzene and 4'Fluoro-4-dimethylaminoazobenzene. *Cancer Research*, 12: 192, 1952.
11. Richardson, H. L., and E. Borsos-Nachtnebel: Study of Liver Tumor Development and Histologic Changes in Other Organs in Rats Fed the Azo Dye 3'Methyl-4-dimethylaminoazobenzene. *Cancer Research*, 11: 398, 1951.
12. Schneider, W. C., and V. R. Potter: The Assay of Animal Tissues for Respiratory Enzymes. II. Succinic Dehydrogenase and Cytochrome Oxidase. *J. Biol. Chem.*, 149: 217, 1943.
13. Striebich, M. J., E. Shelton, and W. C. Schneider: Quantitative Morphological Studies on the Livers and the Liver Homogenates of Rats Fed 2-Methyl-4-dimethylaminoazobenzene or 3'Methyl-4-dimethylaminoazobenzene. *Cancer Research*, 13: 279, 1953.
14. Weber, G., and A. Cantero: Glucose-6-phosphatase Activity in Normal, Precancerous, and Neoplastic Tissues. *Cancer Research*, 15: 105, 1955.

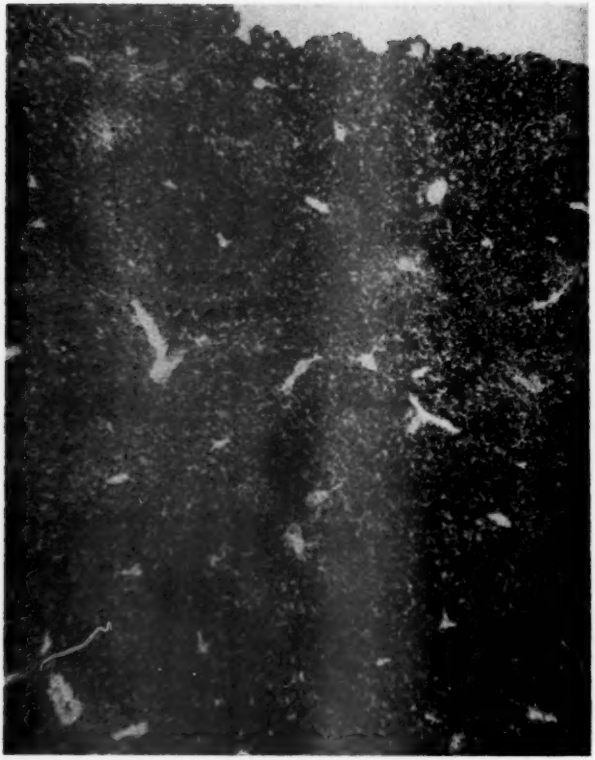


PLATE I

Glycogen storage in the normal control rat liver after two weeks on basal diet. P.A.S. procedure. Rat number A-9. X35.

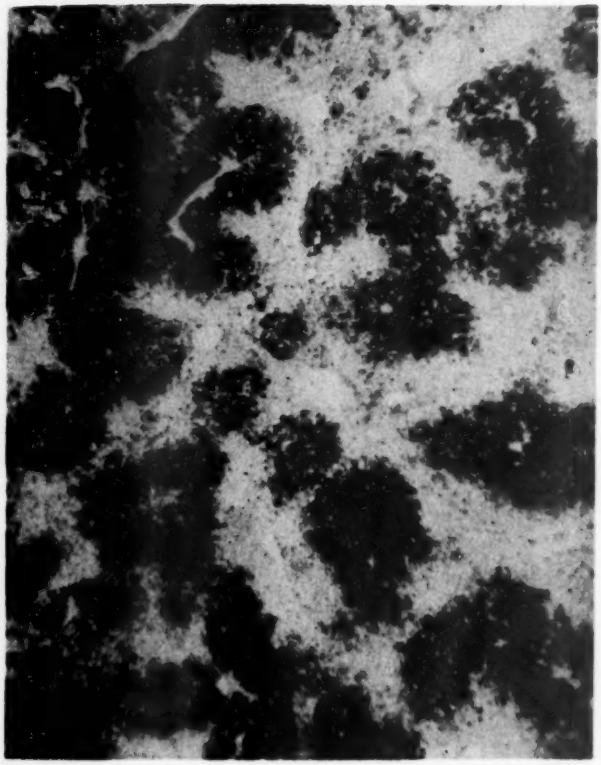


PLATE II

Liver glycogen storage after two weeks treatment with 3'Me-DAB. Notice extensive depletion of glycogen around the central veins with adequate storage in the portal areas. Rat number A-11, pair-fed with A-9 above. P.A.S. X35.

PLATE III

Liver glycogen after two weeks 3'Me-DAB. Higher power showing two central veins and one portal triad. P.A.S. X100.

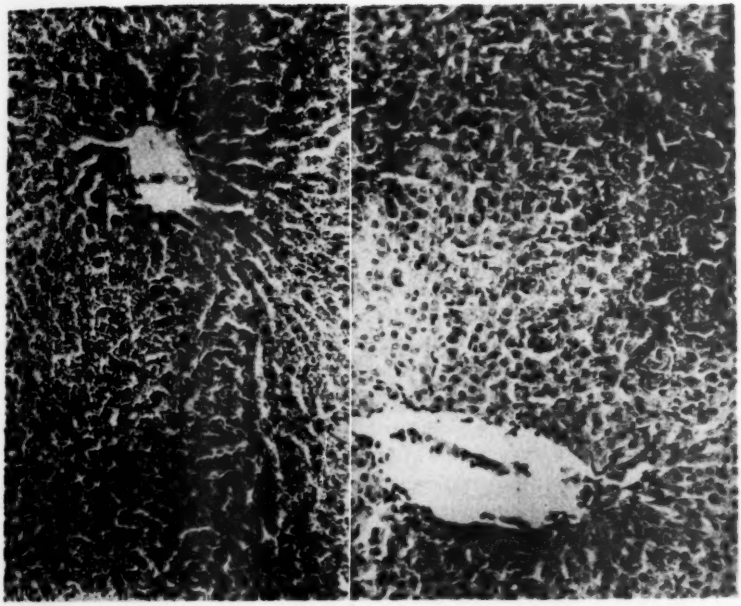


PLATE IV

Cytoplasmic basophilia in the livers of rats following treatment with basal diet (left) and 3'Me-DAB (right) for two weeks. Note the lack of normal granular appearance in a wide area surrounding the central vein following 3'Me-DAB treatment. Pyronin stain. X150.

ANTICANCER ACTIVITY OF PLANT EXTRACTS

ALFRED TAYLOR, GEORGE F. MCKENNA, AND
HENRY M. BURLAGE*

There are, as yet, no particularly promising leads in the field of cancer chemotherapy. Various compounds do adversely affect certain malignant growths but at the same time are so toxic to nontumor tissues as to be of limited value in clinical work. It is obvious that a really successful therapy must involve an agent which is selectively inimical to tumor tissue. The possibility of discovering chemicals with such properties is, of course, the motivation back of the screening programs maintained in many laboratories devoted to cancer research.

Our knowledge in this field is still so limited that it is difficult to make a rational approach to the problem or to justify the testing of particular groups of compounds as compared to other source materials. There is no doubt but that cancer etiology concepts influence cancer therapy research. If the tumor process is the results of irreversible changes in cell biochemistry then the chemotherapeutic agent must be designed to act on the cancer cell through the biochemical characteristics which are associated with malignant growth. At the present time much research is concerned with the attempt to reveal differences in tumor cell chemistry which could be used as a basis for a chemotherapy. As yet, if there are such chemical specificities correlated with a tissue malignancy, they have not been disclosed. However, if cancer is due to the reaction of the cell to a tumor agent, then the biochemistry of cancer tissue would likely differ only quantitatively from that of noncancer tissue and a more empirical approach to tumor therapy would be in order. Under such circumstances, the use of naturally occurring compounds would have as much in its favor as the testing of compounds tailored to accomplish particular effects in the cell physiology.

If we can apply to cancer chemotherapy the experience gained in the efforts to control various diseases of bacterial origin, then

* From the Biochemical Institute and Pharmaceutical Foundation of The University of Texas and the Clayton Foundation for Research, Austin, Texas.

certainly the investigation of compounds associated with organic materials offers a more hopeful approach. The sulfa drugs played their part in combatting infectious diseases, but the antibiotics derived from ready made compounds have proved far more useful since their action is selective and comparatively nontoxic.

Plants especially would seem to be worth investigating in this respect since this group of organisms has yielded so many potent medicines in the past.

These were some of the considerations which led to the initiation, at The University of Texas about six years ago, of a project for screening plant extracts for possible anticancer activity. It was begun as a small operation and has gradually been increased to its present status. The work is still on a moderate scale but is of sufficient scope to reveal something of the possibilities of plant products in cancer therapy.

The techniques associated with various aspects of the study have been modified and improved in accordance with special problems that have been encountered. This has involved the development of two different procedures. One set of manipulations has been concerned with the preparation and preservation of the plant fractions and the other with the testing of extracts for their effects on tumor and nontumor tissues.

From the beginning we considered it important to process the plants in such manner as to preserve the extracts as much as possible in their original state. This has proved to be difficult since many plant fractions contain such a variety of compounds that even at low temperatures, there is likely to be considerable chemical activity. As a result, the antitumor effects obtained with freshly prepared extracts may disappear rapidly unless special procedures are used to prevent it.

Testing of materials for possible adverse tumor effects had been routine in the University Biochemical Institute cancer research laboratories for a number of years prior to the commencement of the plant investigation. A technique had been developed which involved the use of yolk sac inoculated tumor bearing eggs (Taylor *et al.*, 1942; Taylor *et al.*, 1943; Taylor *et al.*, 1948; Taylor, 1953). This method makes it possible to assay rapidly various materials for their anticancer properties and at the same time gives data on the reaction to the test compound of growing embryo tissue. Preliminary evaluations of tumor growth inhibiting effects

can be made in 24 to 48 hours and the cost and effort is much less than that required where animal transplants are utilized (Taylor and Carmichael, 1948; Taylor, 1953a). In addition to eggs bearing tumors, transplants of a mammary carcinoma and a lymphosarcoma in mice have been included in the testing program (Taylor and Carmichael, 1953).

This report is concerned with data obtained from the work done with nearly 1,000 plant extracts including 107 species of plants. The results obtained with some of these plants have been reported previously (Taylor *et al.*, 1951; Taylor *et al.*, 1952; McKenna *et al.*, 1954; McKenna *et al.*, in press).

When the project was initiated, plants for processing were selected on the basis of known biological effects. Plants with recognized toxic properties were considered more likely to yield extracts adverse to tumor growth, since biologically active fractions would be present (Taylor *et al.*, 1952; McKenna *et al.*, 1954). In the experience gained by actual testing of these and other plant types it became evident that nontoxic plants may be a source of growth-inhibiting substances. For example, tumor growth has been retarded by cucumber juice and extracts of a muskmelon. Also, as would be expected, different parts of a plant may differ in extract potentialities. The seeds of plants alone constitute an almost untouched field in this respect. We have barely begun to test plant seed extracts and have noted growth inhibitors in fractions of alfalfa and beet seeds.

Experimental Procedures

Preparation of extracts. The fresh material was processed as soon as possible after collection, and usually the whole plant was extracted as a conglomerate. Dried material was obtained from various drug companies. All material was placed in a Waring Blendor with the solvent. The action comminuted the fresh material, mixed, and wetted it thoroughly. Dried powders were easier to wet when treated in this manner. The first extracts were made with 100 gms. of plant material with the following solvents: water at pH 7.0, pH 6.0, pH 8.0, water at pH 7.0 and boiled 15 minutes, water at pH 7.0 and incubated 24 hours at 37° C., acetone, and absolute alcohol.

Extractions were carried out at as low a temperature as possible. Extracts, except the one incubated, were stored seven days

in a refrigerator. The extract incubated 24 hours was stored six days. The storage was to allow the more slowly soluble materials to go into solution. After storage, the extracts were filtered (Whatman No. 1 paper) and the liquid was freeze-dried. The dried material was dissolved in the least amount of triple distilled water. Acetone extracts will usually dissolve in water but at a lower concentration. The water extracts were sterilized by filtering through a Selas No. 2 porcelain filter. Alcohol extracts were sterilized by placing the extract in a 25 or 50 ml. Erlenmeyer flask with a small quantity of 70 per cent alcohol. The flask was placed in a desiccator that had been rinsed with 70 per cent alcohol, and the alcohol evaporated by use of water pump or vacuum pump. Acetone extracts were sterilized in the same manner as the alcohol extracts except that acetone was used in place of 70 per cent alcohol.

Extracts were tested for sterility by drawing out a sample with a pipette and placing it in a bottle of semi-solid brain-heart infusion media. Occasionally, the extract reacted with the media, in which case a small amount was placed on a nutrient agar plate or into nutrient broth. An extract was considered sterile if no growth was observed after 48 hours.

Methods of anticancer evaluations. The plant fractions were tested against both egg cultivated tumors and tumors transplanted in mice. The sterile extracts were first checked for toxicity by trials with mice or embryonated eggs.

In mice, subdermal injections of various concentrations were made and the effect on weight and visceral organs noted. In toxicity tests with eggs, injections of the test material were made over the blood vessels of the area vasculosa of the chick embryos on the 11th day of incubation. On the 16th day of incubation, the embryos were harvested and weighed. Toxicity was evaluated on the basis of weight and survival as compared with controls receiving saline injections. In most instances, the toxicity range was comparable for mice and 11- to 16-day chick embryos. In preliminary evaluations against tumors, near maximum tolerated dosages were used.

The method used in testing extracts against egg cultivated tumor was similar to that described in previous reports (Taylor, 1953a, 1953b). Embryonated eggs were yolk sac-inoculated through the air sac on the 4th day of incubation with a saline sus-

pension of dispersed tumor tissue. A C_3H mouse mammary carcinoma especially adapted to growth by this type of cultivation was used. The test extract was injected over the area vasculosa of the tumor bearing eggs on the 10th to the 12th day of incubation or 6 to 8 days after the eggs were inoculated with the mouse tumor tissue. At this stage the tumors averaged 0.1 to 0.2 gms. in size depending on whether the 10 or 12 day eggs were used.

The technique of introducing the extract into the eggs is quite simple. A small dent is made in the egg shell with the sharp point of a blade of a pair of small scissors, at a spot, as determined by candling, that is over the blood vessels of the area vasculosa but below the embryo. The dent is small enough so that the point of a 22-gauge hypodermic needle can be inserted and with a little rotation the needle can be pushed through the shell. The needle is inserted at right angles to the shell surface and sent in just beyond the needle bevel. The plunger of the syringe is then pressed in, expelling the contents (0.2 cc.) into a space immediately under the shell membrane and in contact with the yolk sac blood vessels. With very little practice the operation can be done rapidly and effectively. There is no tendency for regurgitation when the dent in the shell is smaller than the hypodermic needle.

The material injected in this manner passes into the blood stream of the chick embryo and is carried to the yolk sac implanted tumor. Numerous experiments have demonstrated that the embryos of eggs injected with saline solution according to this procedure will continue their growth, survival and development exactly as if they were untreated. This may be important since it demonstrates that the operation is not a factor in the reaction of tumor or embryo to the injected material.

Groups of about 15 eggs were utilized for the testing of each extract. A group of 20 or more controls received distilled H_2O. The effect of the plant fraction was based on weight differences in the tumors and embryos of the experimental eggs as compared with the controls.

In some experiments, solutions of the plant extracts were mixed with cottonseed oil to slow down the rate of adsorption, but since there was a possibility that the oil might react with some of the extracts, it was discontinued. Experiments with compounds known to inhibit tumor growth indicated that a marked inhibit-

ing capacity would be manifested even though rapidly absorbed and passed through the embryo blood system. The egg method of assaying antitumor activity of materials was regularly checked by trial and triethylene melamine. This compound consistently inhibits the growth of the C_3H mammary carcinoma used in the egg work. Generally, extracts which failed to inhibit tumor growth 60 per cent or more for the period of the test without serious effect on the embryo were discarded.

For the tests against tumors cultivated in mice, a dba mouse mammary carcinoma and a lymphosarcoma were used. The mammary tumor developed spontaneously in breeding stock in 1941, and has been carried since that time by transplants in mice and eggs. It is known under the designation of RC mammary adenocarcinoma and has been used in screening programs in other laboratories (Tarnowski and Stock, 1955).

Transplants of this tumor in the dba strain maintained in the Biochemical Institute mouse colony, grow rapidly attaining a size of about 4.0 gms. by the 14th day following implantation with no regressions. The lymphosarcoma (dba lymphosarcoma 150) used in these tests arose spontaneously in the inguinal lymph node of a dba mouse several years ago and has been maintained by transplantation in mice. This tumor grows to about 3.0 gms. in size by the 14th day with no tendency for regression.

Transplants of both tumors were made by subdermal injections (1 cc. tuberculin syringe) in the right inguinal area of 0.02 cc. of tumor tissue dispersed in 0.2 cc. of 0.85 per cent saline solution. The mice were divided into equivalent control and experimental groups. The controls received in the left inguinal area an injection of 0.2 cc. distilled H_2O. The experimental mice were given one injection of the test extract at or near the maximum tolerated concentration for the preliminary test. Ten days later, the mice were killed and the tumors weighed. Body weight changes were recorded. The effect of the plant fraction was evaluated on the basis of the difference in the average weight of the tumor from the experimental groups as compared with the controls. If the extract was associated with a 40 per cent or more inhibition of tumor growth, with little or no effect on body weight, further tests were completed.

TABLE 1

The Effect of Plant Extracts on the Growth of Tumor and Embryo of Tumor-Bearing Eggs, 24-Hour Test Period

Plant	Type of Extract	No. Expts.	No. Eggs	Dosage	Survival, Control = 100	Aver. Growth for Test Period, Control = 100	
						Tumor	Embryo
Alfalfa Seed for Beverage	pH 7 Inc.	3	127	3.0 mg.	88	4	121
	pH 7 Boiled	2	80	3.0 mg.	100	30	66
Anemone decapetala Ard.	Absolute Alcohol	2	89	1.5 mg.	100	15	105
Arachis hypogaea L. var. Valencia	pH 7	3	126	3.0 mg.	82	33	85
Arundo Donax L.	Absolute Alcohol	2	82	4.5 mg.	100	29	54
Cascara Sagroda Bark	pH 7	2	89	12.0 mg.	100	26	120
Cooperia pedunculata Herb.	Acetone	2	85	12.0 mg.	100	15	101
Cucumis Melo L. var. Commercial Muskmelon	Acetone	2	64	1.5 mg.	100	27	108
Cucumis sativus Linn-Commercial var. Hasting's Model Cucumber	Expressed Juice	3	131	12.0 mg.	98	23	126
Cucurbita Pepo Linn-Commercial var. Hasting's Jumbo Pumpkin	Expressed Juice	2	87	12.0 mg.	100	40	146
Nerium Oleander L.	pH 7	2	90	1.5 mg.	100	39	154
Prosopis glandulosa Torr.	Absolute Alcohol	2	27	0.06 mg.	100	45	118
	Absolute Alcohol	2	27	0.1 mg.	100	22	105
			32	1.0 mg.	93	36	100
Prosopis glandulosa Torr.	pH 6.0		43	12.0 mg.	62	34	102

	Alcohol	2	32	1	1.0 mg.	95	56	100
Prosopis glandulosa Torr.	pH 6.0		43		12.0 mg.	62	34	102

TABLE 2

The Effect of Subdermal Injections of Plant Extracts in DBA Mice Bearing Transplant Tumors. 10-Day Test Period

Plant	Type of Extract	No. Expts.	No. Mice	No. Inj.	Dosage per Inj.	Body wt. at Autopsy, Control =100	Tumor Growth at Autopsy, Control =100	Type of Tumor
Albizi Julibrissin Durazzini	pH 7.1	1	12	5	6 mg.	101	52	RC mammary carcinoma
		1	12	1	12 mg.	88	55	RC mammary carcinoma
Alfalfa Seed	pH 6.0	3	27	1	12 mg.	102	50	RC mammary carcinoma
	pH 8.0	3	27	1	12 mg.	103	49	RC mammary carcinoma
Arachis hypogaea L. var. Valencia	Absolute Alcohol	1	9	1	21 mg.	99	41	RC mammary carcinoma
		2	18	1	18 mg.	100	43	RC mammary carcinoma
Beta vulgaris Linn-Commercial var. Jumbo Mango Beet	pH 7.0 Boiled	2	18	1	12 mg.	101	48	RC mammary carcinoma
Conium maculatum L.	Acetone	2	24	1	30 mg.	104	51	RC mammary carcinoma
Croton monanthogynus Michx.	pH 7.0	1	9	1	21 mg.	95	51	RC mammary carcinoma
		1	9	1	18 mg.	106	28	RC mammary carcinoma
Cucumis Melo L. var. Staley's Best	Acetone	2	18	1	16 mg.	99	42	RC mammary carcinoma
	pH 7.0	2	18	1	18 mg.	109	31	RC mammary carcinoma
		3	27	1	21 mg.	99	43	RC mammary carcinoma
	pH 7.0 Boiled	2	18	1	18 mg.	107	48	RC mammary carcinoma
	pH 7.0 Inc.	2	18	1	18 mg.	104	42	RC mammary carcinoma
	pH 8.0	4	36	1	21 mg.	101	44	RC mammary carcinoma
Cucurbita foetidissima HBK	Expressed Liquid from Plant	2	24	4	.1 ml.	92	47	RC mammary carcinoma

TABLE 2—Continued

The Effect of Subdermal Injections of Plant Extracts in DBA Mice Bearing Transplant Tumors. 10-Day Test Period

Plant	Type of Extract	No. Expts.	No. Mice	No. Inj.	Dosage per Inj.	Body wt. at Autopsy, Control = 100	Tumor Growth at Autopsy, Control = 100	Type of Tumor
Cyamopsis tetragonoloba† (Indian var.) (seed)	Absolute Alcohol	2	18	1	12 mg.	105	51	RC mammary carcinoma
Liatris punctata Hook	pH 8.0	1	12	1	1 mg.	98	52	RC mammary carcinoma
		1	12	1	4.5 mg.	99	25	RC mammary carcinoma
Licorice Root U.S.P.	pH 7.0 Boiled	2	18	1	18 mg.	102	44	RC mammary carcinoma
Red Cinchona Bark N.F.	pH 6.9	2	18	1	18 mg.	105	32	RC mammary carcinoma
Senna U.S.P.	pH 8.0	2	24	5	1.5 mg.	111	50	RC mammary carcinoma
		1	12	5	4.5 mg.	101	52	RC mammary carcinoma
Wild Cherry Bark U.S.P.	pH 6.0	2	18	1	21 mg.	98	46	RC mammary carcinoma
Cactus	Expressed Juice	2	18	1	96 mg.*	98	41	RC mammary carcinoma
Albizia Julibrissin Durazzini	pH 7.1	2	9	1	12 mg.	92	43	Lymphosarcoma 150
Horse Chestnut Bark	pH 8.0	2	18	1	21 mg.	98	42	Lymphosarcoma 150
Peppermint American U.S.P.	pH 8.2	2	18	1	12 mg.	99	48	Lymphosarcoma 150
Tillandsia usneoides L.	pH 8.0	2	18	1	1.5 mg.	93	53	Lymphosarcoma 150

† A variety from India and a domestic one were tested.
* Based on processors label.

TABLE 3

List of Plants Used in Tumor Chemotherapy Tests

One or more extracts of the following plants inhibited egg cultivated tumor 60 percent to 80 percent in two or more tests:

Albizia Julibrissin Durazzini
Alfalfa Seed for Beverage
Amianthemum muscaetoxicum Gray
Cloves N.F. Powdered[1]
Cyamopsis tetragonoloba[2] (seed)
Datura Stramonium[3] L.
Ergot N.F.[4] (European) (New Crop)
Horse Chestnut Bark[1]
Lupinus texensis Hook
Maclura pomifera (Raf.) Schneid.
Opuntia Mill. sp.
Phoradendron flavescens (Pursh) Nutt.
Rhubarb Root U.S.P.[1] (Powdered)
Sarsaparilla[1]
Sarsasapogenin[5]
Solanum rostratum Dunal
Spearmint[1] (Leaves)
Zephyranthes texana Herb.

One or more extracts of the following plants inhibited egg cultivated tumor 40 percent to 60 percent in two or more tests:

Acalypha phleoides Cav.[6]
Arachis hypogaea L. var. Valencia
Astragalus Wootoni Sheldon[7]
Beta vulgaris Linn-Commercial var. Perfected Detroit Beet (Seed)
Conium maculatum L.[8]
Cucumis Melo L. Linn-Commercial var. Staley's Best
Cucurbita Pepo L.-Commercial var. Hasting's Jumbo Pumpkin
Henbane[1] (Leaves)
Leptotaenia dissecta Nutt. or *Leptotaenia multifida* Nutt.[9]
(July collection)
Liatris punctata Hook
Liucpiaplata o Cola de Caballo[6]
Phoradendron flavescens (Pursh) Nutt.

One or more extracts of the following plants inhibited egg cultivated tumor 40 percent or more:

Alfalfa Seed for Beverage
Arachis hypogaea L. var. Valencia
Arundo Donax L.
Cascara Sagrada U.S.P.[1] (Bark)
Cucumis Melo L. var. Commercial Muskmelon
Cucumis sativus Commercial var. Hasting's Model Cucumber
Cucurbita Pepo-Commercial var. Hasting's Jumbo Pumpkin
Nerium Oleander L.[11]
Results previously published:
Alpha conidendrol[4] and [10]
Anemone decapetala Ard.
Carica papaya Linn
Cooperia pedunculata Herb.
Cucurbita foetidissima HBK
Datura sp.
Prosopis glandulosa Torr.
Purple Sage

† A variety from India and a domestic one were tested.
• Based on processors label.

TABLE 3—Continued

Rhus glabra L.
Rhus Toxicodendron L.
Ricinus communis L.
Salvia Greggii A. Salvia sp. Gray
Yucca arkansana Trel.

Plants with little or no inhibition of egg cultivated tumor:

Aletes acaulis (Torr) C. & R.
Allium Helleri Small
Ambrosia aptera D. C.
Amphyteryngium abstringens[6] (Schlect.) Schiede
Asclepiodora decumbens Nutt.
Astragalus Earlei Greene[7]
Beta vulgaris Linn-Commercial var. Jumbo Mango Beet (Seed)
Campsis radicans (L.) Seem
Cannabis sativa L.
Cassia Tora L.
Cichorium Intybus L.
Coprinus
Croton monanthogynus Michx.
Cucumis Melo Linn. var. cantalupinses
Cucumis Melo Linn var. culta
Cucurbita Pepo Linn-Commercial var. Southern Field Pumpkin
Cucurbita Pepo Linn-var. Ovifera
Delphinium Ajacis[1] (Seed)
Elm Bark N. F.[1]
Eupatorium urticaefolium Reichard
Gossypium sp.
Gutierrezia texana T. & G.
Helianthus annuus L.
Lagenaria leucantha Rusby-Commercial var. Vaughan's Large Fruited Hercules Club
Lagenaria leucantha Rusby-Commercial var. Vaughan's Small Fruited Spoon Gourd
Leptotaenia dissecta Nutt. or *Leptotaenia multifida* Nutt. (June collection)[9]
Licorice Root U.S.P.[1]
Parkinsonia aculeata L.
Parthenium Hysterophorus L.
Peppermint American U.S.P.
Piscaria
Poinciana pulcherrima L.
Red Cinchona Bark N.F.[3]
Salvia farinacea Benth
Senna U.S.P.[1]
Sophora secundiflora (Ortega) Lag.
Tillandsia usneoides L.
Tryamine[12]
Verbena bipinnatifida Nutt.
Viscum album L.[3]
Vitis candicans Engelm.
Wild Cherry Bark U.S.P.[1]
Zea mays L.-Commercial var. Yellow Dent

[1] Contributed by S. B. Penick & Co.
[2] Contributed by General Mills.
[3] Contributed by Lloyd Brothers.
[4] Contributed by U.S.D.A.
[5] Contributed by Heifetz & Co.
[6] Contributed by a Mexican Herbalist.

TABLE 3—Continued

[7] Contributed by Dr. J. W. Dollahite, Texas Agr. Expt. Sta., Marfa, Texas.
[8] Contributed by Mr. Jack L. Groff, County Agent in Bandera County, Texas.
[9] Contributed by Dr. Jos. H. Robertson, Dept. of Range Management, Col. of Agriculture, the Univ. of Nevada, Reno, Nevada.
[10] Contributed by Crown Zellerbach Corp.
[11] Contributed by Mr. M. S. Spooner, Gonzales, Texas.
[12] Contributed by Dr. Greathouse.

TABLE 4

List of Plants Used in Chemotherapy Tests

One or more extracts of the following plants inhibited RC mammary carcinoma transplanted in mice 60 percent to 80 percent in two or more tests:

Aletes acaulis (Torr.) C. & R.
Amphyteryngium abstringens (Schlect)[1] Schiede
Anemone decapetala Ard.
Astragalus Wootoni Sheldon[2]
Berberis Aquifolium Nutt.[3]
Beta vulgaris Linn-Commercial var. Perfected Detroit Beet (Seed)
Cascara Sagrada Bark U.S.P.[4]
Gossypium sp.
Licorice Root U.S.P. (Sarcoma and Carcinoma)[4]
Passiflora incarnata L.[3]
Phoradendron flavescens (Pursh) Nutt.
Rhus Toxicodendron L.
Spearmint (Leaves)[4]
Tillandsia usneoides L.
Verbena bipinnatifida Nutt.
Viscum album L.[3]

One or more extracts of the following plants inhibited RC mammary carcinoma transplanted in mice 50 percent to 60 percent in two or more tests:

Allium Helleri Small
Arundo Donax L.
Cucumis Melo Linn var. Cantalupinses
Cucurbita Pepo Linn var. ovifera[4]
Delphinium Ajacis (Seed)[4]
Henbane (Leaves)[4]
Maclura pomifera (Raf.) Schneid.
Opuntia Mill. sp.
Zea mays L.-Commercial var. Yellow Dent

One or more extracts of the following plants inhibited RC mammary carcinoma transplanted in mice 50 percent:

Alfalfa Seed for Beverage
Arachis hypogaea L. var. Valencia
Beta vulgaris Linn-Commercial var. Jumbo Mango Beet (Seed)
Cactus[3]
Croton monanthogynus Michx.
Cucumis Melo L. var. Staley's Best
Cyamopsis tetragonoloba India var.[5] (Seed)
Licorice Root U.S.P.[4]
Red Cinchona N.F.[3] (Bark)
Senna U.S.P.[4]
Wild Cherry U.S.P.[4] (Bark)

Results previously published:

Albizia Julibrissin Durazzini
Aletes acaulis (Torr.) C. & R.

TABLE 4—Continued

Alpha conidendrol[6] and [7]
Conium maculatum L.[8]
Cucurbita foetidissima HBK
Liatris punctata Hook
Melia Azedarach L.
Parkinsonia aculeata L.
Phoradendron flavescens (Pursh) Nutt.
Prosopis glandulosa Torr.
Rhubarb U.S.P.[4] (Powdered Root)

One or more extracts of the following plants inhibited lymphosarcoma 150 transplanted in mice 50 percent (results previously published):

Horse Chestnut Bark[4]
Peppermint U.S.P. (American)
Tillandsia usneoides L.

Results previously published:

Albizia Julibrissin Durazzini
Cooperia pedunculata Herb.
Croton monanthogynus Michx.

Plants with little or no effect with either RC mammary carcinoma or lyphosarcoma transplanted in mice:

Acalypha phleoides Cav.[1]
Acocotillo o Feuogreco[1]
Ambrosia aptera DC
Amianthemum Muscaetoxicum (Gray)
Anemone Pulsatilla L.
Asclepiodora decumbens Nutt.[2]
Astragalus Earlei Greene[2]
Berberis[3] (Root)
Brauneria angustifolia or *Brauneria pollida*[3]
Campsis radicans (L.) Seem
Cannabis sativa L.
Cassia Tora L.
Chelidonium majus L.[3]
Chionanthus virginica L.[3] (Root)
Cimicifuga racemosa Nutt.[3]
Cloves N.F.[4] (Powdered)
Cnidoscolus tezana (Muell. Arg) Small
Collinsonia canadensis[3] (Root)
Cooperia Drummondii Herb.
Coprinus
Cucumis Melo L.-Commercial var. Staley's Best
Cucumis Melo Linn var. culta
Cucumis sativus Linn-Commercial var. Hasting's Model Cucumber
Cucurbita Pepo Linn-Commercial var. Hasting's Jumbo Pumpkin
Datura sp.
Datura Stramonium L.[3]
Dioscorea villosa L.[3]
Elm Bark N.F.[4]
Ergot N.F.[6] (European) (New Crop)
Eupatorium urgicaefolium Reichard
Euphorbia marginata Pursh.
Gelsemium sempervirens Ait.[3]
Geranium maculatum L.[3]
Gutierrezia tezana T. & G.
Helianthus annuus L.

TABLE 4—Continued

Hydrangea arborescens L.[3]
Ibervillia Lindheimeri (A. Gray) Greene
Liucipiaplata o Cola de Caballo[1]
Lobelia inflata L.[3] (Seed)
Lupinus texensis Hook
Lycopus virginicus[3]
Melia azedarach L.
Nerium Oleander L.[9]
Nicotiana glauca Graham
Parthenium Hysterophorus L.
Passiflora incarnata L.[3] (Root)
Phytolacca decandra[3]
Piscaria
Piscidia erythrina (L.) Sarg.[3]
Podophyllum peltatum L.
Poinciana pulcherrima L.
Purple Sage
Rhus aromatica[3] (Bark of roots)
Rhus Toxicodendron L.
Ricinus communis L.
Salvia farinacea Benth
Salvia Greggi A. Gray
Salvia sp.
Sanguinaria canadensis L.[3]
Sarsaparilla[4]
Solanum rostratum Dunal
Sophora secundiflora (Ortega) Lag.
Thuja occidentalis L.[3]
Toxicodendron flavescens Pursh.
Viscum album L.[3]
Vitis candicans Engelm
Yucca arkansana Trel.
Zephranthes texana Herb.

[1] Contributed by a Mexican herbalist.
[2] Contributed by Dr. J. W. Dollahite, Texas Agr. Expt. Sta., Marfa, Texas.
[3] Contributed by Lloyd Brothers.
[4] Contributed by S. B. Penick & Co.
[5] Contributed by General Mills.
[6] Contributed by U.S.D.A.
[7] Contributed by Crown Zellerbach Corp.
[8] Contributed by Mr. Jack L. Groff, County Agent in Bandera County, Texas.
[9] Contributed by Mr. M. S. Spooner, Gonzales, Texas.

The authors thank Dr. Benjamin C. Thorp, Professor of Botany, Dr. C. C. Albers, Professor of Pharmacognosy, and Dr. Billie L. Turner, Assistant Professor of Botany, for the identification of many of these plants.

Many plants were received from commercial sources and the authors did not check on identification, time of collection, or storage conditions of processing for distribution.

Results

The results are summarized in tables 1–4. Tables 1 and 3 are concerned with the tests made against tumors cultivated in eggs. Table 1 records 14 extracts from 12 plants which inhibited tumor growth 55 to 96 per cent as compared with the growth of tumor in control eggs. This was accomplished, as the table reveals, in most instances, with no adverse effect on the supporting

chick embryos. Only three of the extracts were associated with average embryo weights which were smaller than those of the control embryos. Table 3 contains a list of all the plants processed for tests with egg cultivated tumors and the results. The first group in this table represents plants which through their extracts inhibited tumor growth 60 to 80 per cent. Some of these were left out of table 1 because the extracts were adverse to the embryo or proved to be too difficult to preserve and are of little value until this factor can be controlled. Table 3 also contains a list of the plants and the antitumor activity of which has been given in previous publications.

The data obtained with tumor transplants in mice are given in tables 2 and 4. Table 2 includes results with plant fractions not previously published which inhibited tumor growth about 50 per cent or more for the period of the tests. A total of 15 plants furnished 18 extracts, one from each of four plants, inhibited the growth of lymphosarcoma 150, 47 to 58 per cent.

In checking over the results obtained with the three tumor types, two of which, the RC carcinoma and the lymphosarcoma 150, were grown in mice, and the C_3H mouse mammary carcinoma cultivated in eggs, including published and unpublished data, it appears that one plant, *Albizia*, furnished extracts effective against all three tumors. Two plants, *Tillandsia* and *Albizia*, were effective for both the RC carcinoma and the lymphosarcoma 150. Three plants, *Cooperia*, horse chestnut and *Albizia*, furnished extracts effective against both lymphosarcoma 150 and the egg cultivated C_3H mouse carcinoma. Seventeen plants gave extracts effective against both the egg and mouse cultivated carcinomas. These are listed below:

Albizia Julibrissin Durazzini
Alpha conidendrol
Anemone decapetala Ard.
Astragalus Wootoni Sheldon
Beta vulgaris Linn-Commercial var. Perfected Detroit Beet (Seed)
Conium maculatum L.
Cucumis Melo Linn var. Cantalupinses
Cucurbita foetidissima HBK
Cucurbita Pepo Linn var. *ovifera*
Liatris punctata Hook
Maclura pomifera (Raf.) Schneid
Opuntia Mill. sp.

Phoradendron flavescens (Pursh) Nutt.
Prosopis glandulosa Torr.
Rhubarb Root U.S.P. Powdered
Rhus Toxicodendron L.
Spearmint (leaves)

Of the 86 plants listed in table 3, 43 or 50 per cent gave one or more extracts which, in various degrees, inhibited the growth of the egg cultivated tumors. This represents about 7 per cent of the extracts made from these plants. The RC carcinoma was used in tests with extracts from 107 plants, 36 of which gave extracts inhibiting tumor growth 50 per cent or more. On the basis of the number of extracts, about 5 per cent gave positive results. Less than 1 per cent of the extracts were effective against lymphosarcoma 150.

Discussion

The literature on cancer chemotherapy research is mainly concerned with negative data. This is necessarily so since there is, as yet, no drug preparation which is of much value in the treatment of the great majority of malignant growths occurring in man or any other animal. It follows therefore that the chief value of reports of work in this field is, first to spare others the time and effort of investigating substances which have proved to be of no value, and secondly, to call attention to approaches to the problem which appears to be worthy of further exporation. The present paper belongs to the second of these two categories. None of the plant extracts processed to date have given results which promise much in the way of a practical application to tumor therapy. Some of them will be investigated further, and may prove worthy of clinical trials, but the chief importance of the data presented is that it indicates the possibility of discovering much more potent material if the resources of the plant kingdom, in this respect, were more thoroughly investigated.

In this connection, it is pertinent to recall the immense scope of the projects which led to the discovery of some of the antibiotics. It was not until penicillin had disclosed the possibilities of the compounds occurring naturally in fungi that investgations were undertaken on the massive scale which is necessary when thousands of samples may be processed before an effective compound is discovered. Needless to say, our results do not constitute

such a clear-cut indication that plants are as likely a source of antitumor compounds as was provided by fungi for the field of antibacterials when penicillin was discovered. Nevertheless, the data presented indicate that plant fractions are a source of tumor growth inhibiting substances which, at effective dosages, do not appear to affect adversely rapidly growing non-tumor tissue. Accordingly if plants were investigated on a scale approaching that used in processing fungi for antibiotics, really effective antitumor compounds might be discovered.

Recently a cooperative research (Gellhorn and Hirschberg, 1955) was undertaken in which a series of 28 compounds, 12 of which were known to have antitumor capacity, were checked against a number of tumors and other biological systems. Among the tumors selected for the tests was the RC carcinoma used in the present investigation. It was reported that only 2 of the 28 compounds were effective against this tumor, inhibiting growth about 60 and 75 per cent (Tarnowski, 1955). In the results given in this report, transplants of the RC carcinoma grown in dba mice were inhibited in growth 50 to 80 per cent by 5 per cent of the extracts made from 36 of the total of 107 plants used in the study. These data indicate that antitumor activity was about as frequent among the plant extracts as it was among selected group of 28 organic compounds when checked against the same tumor by essentially the same procedure. This comparison is not made with the idea that other approaches to the problem are unsound or should not be continued but merely to emphasize the possibilities of the plant world in this respect.

At present, there are few reports on the use of extracts from plant tissues in tumor chemotherapy studies. A fairly extensive literature exists on the use of particular plant fractions and compounds such as colchicine, podophyllin, essential oils, etc., in antitumor tests. But those investigations represent a different approach to the one considered in this paper.

Belkin and Fitzgerald (1952, 1953a, 1953b) (Belkin *et al.*, 1952; Fitzgerald *et al.*, 1953) have reported on the antitumor activity of plants used as cathartics, diuretics, pesticides, and other plants. They obtained some promising results. No other reports of whole plant processing for antitumor effects have been noted.

The investigation of raw plant materials as a source of compounds effective in tumor chemotherapy has barely begun. It is

hoped that there will be much more research in this field in the future. On the basis of our experience, we consider that it constitutes a reasonable and hopeful approach to the problem of cancer control.

Summary

For the past 6 years, a project of checking plants for antitumor activity has been maintained at The University of Texas.

A series of nearly a thousand extracts from a hundred plant species has been studied. The extracts were tested against a C_3H mouse mammary carcinoma cultivated in eggs by the yolk sac method, and the RC mammary carcinoma and lymphosarcoma 150 grown as transplants in mice.

The results, in addition to those previously published, were as follows:

Nine plants gave 11 extracts which inhibited the growth of the egg cultivated tumor 55 to 96 per cent without affecting the supporting chick embryos.

Fifteen plants furnished 18 extracts which inhibited the growth of the RC carcinoma 50 to 68 per cent.

Four extracts, one from each of 4 plants, inhibited the growth of the lymphosarcoma 150, 47 to 58 per cent.

In the total results of this research, one plant was effective against all three tumors, 2 plants were effective for both the RC carcinoma and the lymphosarcoma, 3 plants inhibited the growth of the egg cultivated tumor and the lymphosarcoma, and 17 plants furnished extracts effective against the RC carcinoma and the egg cultivated carcinoma.

It is suggested, on the basis of these results, that the exploration of plant chemical resources constitutes a reasonable and hopeful approach to cancer chemotherapy.

Acknowledgements

We wish to extend our appreciation and thanks to the Clayton Foundation for Research for the generous support given this investigation from its inception to the present.

REFERENCES

Belkin, M., and D. B. Fitzgerald: Tumor-Damaging Capacity of Plant Materials. I. Plants Used as Cathartics. *J. Nat. Cancer Inst.*, 13: 139–155, 1952.

Belkin, M., and D. B. Fitzgerald: Tumor-Damaging Capacity of Plant Materials. III. Plants Used as Pesticides. *J. Nat. Cancer Inst.*, 13: 889–893, 1953a.

Belkin, M., and D. B. Fitzgerald: Tumor-Damaging Capacity of Plant Materials. V. Miscellaneous Plants. *J. Nat. Cancer Inst.*, 13: 607–625, 1953b.

Belkin, M., D. B. Fitzgerald, and M. D. Felix: Tumor-Damaging Capacity of Plant Materials. II. Plants Used as Diuretics. *J. Nat. Cancer Inst.*, 13: 741–744, 1952.

Fitzgerald, D. B., M. Belkin, M. D. Felix, and M. K. Carroll: Tumor-Damaging Capacity of Plant Materials. IV. Conifers. *J. Nat. Cancer Inst.*, 13: 895–903, 1953.

Gellhorn, A., and E. Hirschberg, Editors: Investigation of Diverse Systems for Cancer Chemotherapy Screening. *Cancer Res. Suppl. III*, 125, 1955.

McKenna, G. F., A. Taylor, and H. M. Burlage: Chemotherapy Experiments with Plant Extracts and Transplantable Tumors. *Tex. Rept. Biol. and Med.*, 12: 500–508, 1954.

McKenna, G. F., A. Taylor, and H. M. Burlage: The Use of Plant Extracts in Chemotherapy. *Drug Standards*, (in press).

Tarnowski, G. S.: IV. Tumor Inhibition Studies on RC Mammary Adenocarcinoma. *Cancer Res. Suppl. III*, 28–31, 1955.

Tarnowski, G. S., and C. C. Stock: Selection of a Transplantable Mouse Mammary Carcinoma for Cancer Chemotherapy Screening Studies. *Cancer Res.*, 15: 227–232, 1955.

Taylor, A.: The Yolk Sac Method of Cultivating Tumor Tissue in Eggs. *Univ. Tex. Pub. 5314, Biochem. Inst. V*, 13–26, 1953a.

Taylor, A.: The Use of Embryonated Eggs in Cancer Therapy Experiments. *Univ. Tex. Pub. 5314, Biochem. Inst. V*, 27–35, 1953b.

Taylor, A., and N. Carmichael: Yolk Sac Cultivated Tumor Tissue and Experiments in Tumor Chemotherapy. I. Riboflavin. *Tex. Rept. Biol. and Med.*, 6: 504–512, 1948.

Taylor, A., and N. Carmichael: The Effect of Metallic Chlorides on the Growth of Tumor and Nontumor Tissue. *Univ. Tex. Pub. 5314, Biochem. Inst. V*, 36–79, 1953.

Taylor, A., J. Thacker, and D. Pennington: Growth of Cancer Tissue in the Yolk Sac of the Chick Embryo. *Science*, 96: 342–343, 1942.

Taylor, A., R. E. Hungate, and D. R. Taylor: Yolk Sac Cultivation of Tumors. *Cancer Res.*, 3: 437–441, 1943.

Taylor, A., N. Carmichael, and T. Norris: A Further Report on Yolk Sac Cultivation of Tumor Tissue. *Cancer Res.*, 8: 264–269, 1948.

Taylor, A., N. Carmichael, G. F. McKenna, and H. M. Burlage: Inhibition of the Growth of Egg Cultivated Tumor Tissue by Extracts of *Cooperia pedunculata* Herb. *Proc. Soc. Exper. Biol. and Med.*, 77: 841–843, 1951.

Taylor, A., G. F. McKenna, and H. M. Burlage: Cancer Chemotherapy Experiments with Plant Extracts. *Tex. Rept. Biol. and Med.*, 10: 1052–1074, 1952.

SOME ASPECTS OF THE CHEMISTRY OF THE PITUITARY GROWTH HORMONES OF DIFFERENT SPECIES

A. E. WILHELMI
Biochemistry Department, Emory University
Emory, Georgia

Observations have been collected over a period of years on the chemical, physical and biological properties of anterior pituitary growth hormone preparations from ox, pig, sheep, horse, and fish of two species, hake and pollack. It is hoped also to have preliminary information on a preparation from monkey pituitaries. Data will be presented on relative yields, solubility, isoelectric behavior, biological activity, sedimentation in the ultracentrifuge, N-terminal groups, and certain other properties of growth hormone preparations of comparable orders of purity. The evidence indicates that there are differences, in molecular weight and in isoelectric point, at least, between some of the pitutitary growth hormones, and that, as between mammalian and fish growth hormones, there may be fundamental differences in biological activity.

CALLING ATTENTION TO

Items of Possible Interest to Medicos

June, 1956

General: JM Brown looks *Through These Men* at our great contemporaries (Harper, NY, 1956, 302pp. $4). JB Conant examines *The Citadel of Learning* (Yale Univ. Press, New Haven, Conn., 1956, 98pp, $2). E Fromm proposes *The Sane Society* (Rhinehart, NY, 1956, 340pp, $5). O Lewis recalls romantic San Francisco in *Bay Window Bohemia* (Doubleday, NY, 1956, 248 pp, $4). A Maurois does it again in *Olympio: The Life of Victor Hugo* (1801–1884) (Harper, NY, 1956, 370pp., $6. CW Mills reveals *The Power Elite*, the self-perpetuating big brass (Oxford Univ. Press, NY, 1956, 345pp, $6). RM Underhill does justice to *The Navajos* (Univ. Oklahoma Press, Norman, 1956, 276pp., $4.5). M White points *Toward Reunion in Philosophy* (Harvard Univ. Press, 1956, 320pp, $5.75).

History of Medicine: OL Bettman gives *A Pictorial History of Medicine* (CC Thomas, Springfield, Ill., 1956, 331 pp, $10). LA Cohen carefully repeats experiments of Robt Boyle (1627–1691) on survival of mice in jar with lighted candle (*J Hist Med* 11/127/56). WS Cole looks wisely on *Sex in Christianity & Psychoanalysis* (Oxford Univ. Press, NY, 1956, 211pp, $4). SR Cutolo tells the story of a great hospital in *Bellevue Is My Home* (Doubleday, NY, 1956, 273pp, $4). RM Green offers *Asclepiades: His Life and Writings* (E Licht, New Haven, Conn, 1956, 178pp, $6). LS Kubie well discusses Freud & Freedom in *Sat Rev* Freud centennial number of May 5, 1956. CF Mullett tells about *The Bubonic Plague & England*, Univ. Kentucky Press, Lexington, 1956, 620pp, $9). MK Opler relates *Culture, Psychiatry & Human Value* (CC Thomas, Springfield, Ill., 1956, 253pp, $5). FM Rackeman tells about "The Inquisitive Physician: The Life & Times of George R. Minot 1885–1950" (Harvard Univ. Press, Cambridge, Mass, 1956, 300pp, $5). RL Schoenwald is semi-historical in *Freud: The Man and His Mind* (AA Knopf, NY, 1956, 250pp, $4) O Temkin translates *Soranus' Gynecology* (Johns Hopkins Press. Balt., 1956, 308pp, $5). G Tourney shows old faces in new masks in relating Empedocles & Freud, Heraclitus & Jung (*Bull Hist Med* 30/109/56).

Clinical: WD Block & K van Goor consider *Metabolism, Pharmacology & Therapeutic Use of Gold* (CC Thomas, Springfield, Ill., 1956, 76pp, $1.8). B Bronte-Stewart & Co. indicate highly unsaturated fatty acids in diet depress serum cholesterol (*Lancet* 1/521/Apr. 28/56). FJJ Buytendijk discusses *Allgemeine Theorie der menschlichen Haltung und Bewegung* (Lange & Springer, Berlin W35, 1956, 375pp 40, DM). CF Fluhmann describes *The Management of Menstrual Disorders* (WB Saunders, Phila, 1956, 350pp, $8.5). EJ Stieglitz edits symposium on problems of the mind in later life (*Geriatrics* 11/137–179/56). W Wolff shows how well *Contemporary Psychotherapists Examine Themselves* (CC Thomas, Springfield, Ill., 1956, 313pp, $6).

PHYSIOLOGY & PHARMACOLOGY: W de Boer discusses *Pharmakopsychologie und Psychopathologie* (Lange & Springer, Berlin 4 35, 1956, 301pp, 40 DM). US von Euler well discusses *Noradrenaline* (CC Thomas, Springfield, Ill., 1956, 401pp, $8). JF Fazekas & Co. show mutual antagonism of metrazole & depressant barbitals (*Am J Med Sci* 23/531/56). RFW Goodwin indicates high fetal fructose is peculiarity of ungulates (J Physiol 132/146/56). LV Heilbrunn stresses calcium in *The Dynamics of Living Protoplasm* (Academic Press, NY, 1956, 337pp, $8). GW Milton & AWM Smith show pace-maker for duodenum near bile duct opening (*J. Physiol* 132/110/56). J Rolin discourses well on *Police Drugs* (Philosophical Library, NY, 1956, 202pp, $4.75). PH Rossier & Co. analyze *Physiologie und Pathophysiologie der Atmung* (Lange & Springer, Berlin W35, 1956, 337pp, 49 DM). EN Wynder edits *The Biologic Effects of Tobacco* (Ciba Symposium, Little Brown, Boston, 1956, 256pp, $4.5).

C.D.L.

TEXAS REPORTS ON BIOLOGY AND MEDICINE

Volume 14, 1956

Author Index

	Page
Abildskov, J. A.	204
Altenburg, Edgar	481
Anigstein, Ludwik	401
Archer, John D.	1
Aub, Joseph C.	475
Belkin, Gerald A.	415
Blocker, T. G. Jr.	372
Blumel, Johanna	269
Brachetto-Brian, D.	276
Burlage, Henry M.	538
Butler, James Keith	6
Corcoran, Michael C.	292
Cuadra C. Manuel	97
de Chenar, C.	303
Dillon, T.	432
Earle, Kenneth M.	208
Eggers, G. W. N.	6
Emerson, George A.	464
Engley, Frank B., Jr.	10, 114, 313
Evans, E. Burke	6
Fleck, Ludwik	424
Goth, Andres	432
Gregory, Raymond L.	388
Hardin, W. B.	401
Hisey, B. L.	204
Hopps, Howard C.	362
Ingerson, W. E.	204
Johnson, Joseph K.	65
Johnston, James A.	74
Levin, William C.	372
Li, Choh-Luh	208
Luckey, Thomas D.	482

Author Index

Page

May, Lawrence G. 388
McKenna, George 538
Mefferd, Roy B., Jr. 506
Montgomery, P. O'B. 376, 432
Moorhead, P. S. 237

Nowicki, Eugene L. 79

Patterson, Marcel 226
Pavan, Mario 254, 459
Perry, John E. 372
Pomerat, C. M. 237

Rigdon, R. H. 508

Schneider, Rose G. 380
Scott, J. Allen 440
Shapiro, Alvin P. 415
Spain, J. D. 528
Stembridge, Vernie A. 388
Stimmel, Carolyn M. 440

Taylor, Alfred 538
Tsai, Shih Yuan 388

Whitney, Dorothy M. 254, 401, 459
Wilhelmi, A. E. 557

Yoo, Athanasius Eul Joon 464

TEXAS REPORTS ON BIOLOGY AND MEDICINE

Volume 14, 1956

Subject Index

	Page
Adrenal weight in rats	415
Adsorption of sulfur-35	372
Alcohol	1
Allergen-specific enzymatic factor	79
Allergic skin reaction	79
Amino acids in salivary glands	269
Anticancer activity of plant extracts	538
Antithyroid antibodies	401
Bartonellosis, human	97
Behavioral disturbances in animals	415
Blood derivative, Sanguinin	254, 459
Blood pressure in rats	415
Body materials, persistence of microorganisms in	10
Brain anatomy	208
Brain modelling	208
Breast, cancer of	276
Calcium, effect on chromogenesis in Micrococcus Pyogenes	74
Calling Attention to	90, 264, 396, 558
Cancer incidence in the community	292
Cancer of the breast	276
Cancer, radical breast operation for	65
Cancer symposium	472–557
Cancer research is growing up	475
Carcinogenesis, azo dye	528
Carcinogenesis in the white Pekin duck	508
Carcinogens	481
Chromogenesis in micrococcus pyogenes, effect of calcium on	74
Culture media (Persistence of microorganisms in)	114
Dilemmas of psychosomatic medicine	226

Subject Index

Page

Electrophoresis, paper 380
Endamoeba histolytica 464

Food, Persistence of microorganisms in 313

Glycerol on cells, effect of 237

Histamine liberator 432
Histochemical study of pulmonary hyaline membrane disease of infancy 376
Historical aspects of the radical breast operation for cancer 65

Infancy, pulmonary hyaline membrane disease of 276

Knee joint, serially sectioning of 6

Lethal synergism between Secobarbital and alcohol 1
Leukemic mouse, biochemical change in 506
Leukergy 424
Linear Time Scale 204

Medicine, psychosomatic 226
Metabolism in germfree animals 482
Methylcholanthrene 432
Method for serially sectioning the knee joint of a mature rat 6
Methimazole on thyroid of the dog 388
Micrococcus pyogenes 74
Microorganisms, persistence of 10, 114
Mitation rate by carcinogens 481

Paper electrophoresis 380
Parenchymal components, polymetachromatic staining of 303
Pepsinogen in rats 415
Persistence (Survival) of microorganisms 10, 114, 313
Philosophy of research 362

Pituitary growth hormones 557
Polycystic ovary syndrome 276
Polymetachromatic staining 303
Precancerous metabolic alterations 528
Present day status of the radical breast operation for cancer 65
Psychosomatic medicine 226
Pulmonary hyaline membrane disease 376

Subject Index

	Page
Radical breast operation for cancer	65
Radioiodinated antithyroid antibodies	401
Reconstruction of brain anatomy	208
Research, philosophy of	362
Salivary glands, amino acids in	269
Salmonellosis	97
Sanguinin, a blood derivative	254, 459
Schistosoma haematobium	440
Secobarbital	1
Serum proteins *in vitro*	372
Skin carcinoma	432
Society of American Bacteriologists, Texas Branch (fall meeting)	259
Spatial vectocardiographic data on a linear time scale	204
Stromatic components, polymetachromatic staining of	303
Texas Branch, Society of American Bacteriologists (fall meeting)	259
Thermal stress and tissue culture	237
Thiouracil on thyroid of the dog	388
Thyroid of the dog	388
Tissue culture and thermal stress	237